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CLINICAL AND EXPERIMENTAL STUDIES ON COW'S MILK PROTEIN INTOLERANCE IN INFANCY

Iréne Jakobsson

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II N.O. Berg, I. Jakobsson & T. Lindberg:
Do pre- and postchallenge small intestinal biopsies help to diagnose cow´s milk protein intolerance?
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III I. Jakobsson & T. Lindberg:
Cow´s milk as a cause of infantile colic in breast-fed infants.
The Lancet 1978; 2:437-39.

IV I. Jakobsson & T. Lindberg:
Cow´s milk proteins cause infantile colic in breast-fed infants. A double-blind cross-over study.
Pediatrics. Submitted for publication.

V I. Jakobsson, T. Lindberg & B. Benediktsson:
In vitro digestion of cow´s milk proteins by duodenal juice from infants with various gastro-intestinal disorders.
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DEFINITIONS

Allergy — Von Pirquet (136) introduced the concept of allergy defined as a state of changed reactivity in 1906. Since 1966, the term allergy has been used mostly to describe IgE-mediated reactions (56, 60), or sometimes to describe all reactions caused by immunological mechanisms of the four main types described by Coombs and Gell (22).

Cow´s milk protein intolerance — It is still not known how all adverse reactions to cow´s milk proteins are mediated. Therefore we prefer the term cow´s milk protein intolerance, meaning any adverse reaction, whether of immediate or delayed type, to cow´s milk proteins, giving symptoms from the gastro-intestinal tract, the skin, or the respiratory tract.

ABBREVIATIONS

CMPI	Cow´s milk protein intolerance
αLA	α-lactalbumin
βLG	β-lactoglobulin
IgG	Immunoglobulin class G
IgM	Immunoglobulin class M
IgA	Immunoglobulin class A
IgE	Immunoglobulin class E
RAST	Radioallergosorbent test

INTRODUCTION

Adverse effects from foods or drinks are on record from ancient times. Hippocrates observed that cow´s milk could cause gastric upset and urticaria (18). In 1860 Salter (108) published a detailed study noting the significance of inhalants and substances absorbed from the stomach and intestinal tract on the precipitation of asthmatic attacks. He also described a young child whose asthma was brought on by ingestion of cow´s milk. The first years of this century saw some reports in the German literature about infants with adverse reactions to cow´s milk (115, 33), one case of an anaphylactic reaction with death was described (33). Reports ensued in the American literature through Talbot in 1916 (127). He described two cases of "idiosyncrasy" to cow´s milk. Both infants thrived well on goat´s milk. He concluded that foreign proteins might pass through the intestinal wall of infants shortly after birth or in later infancy if the mucous membranes were injured. Finally he stressed the importance of a hereditary predisposition to sensitization.

Before 1950 CMPI was considered a rare disease (133). Since 1950 there has been an increased number of reports on this subject. The manifestations of CMPI have been described (20, 3, 19, 96, 42, 47, 40, 53) with dermatologic, gastrointestinal, respiratory, and central nervous symptoms. Atopic dermatitis occurred in 30-50% (19, 42, 40). Modifications of behaviour have been shown to occur in 18-20% (19, 42). The infants were described as unhappy, uncomfortable, hyperkinetic, hyperirritable. Colic has been reported

as a rather common symptom (19, 42, 40) occurring in 20-30% of infants with CMPI. Vomiting and diarrhoea have been described in 30-50% (42, 40, 53), mostly with an acute onset as in acute infectious gastroenteritis. In 1958 Clein (19) described a few infants who had been thought to have coeliac disease but were relieved of their symptoms when cow´s milk was eliminated from the diet.

Otherwise, this type of CMPI with a slow onset of symptoms resulting in chronic diarrhoea and malabsorption combined with small intestinal mucosal damage was not described until 1963 (75). Since 1965 (38, 67, 68) increased attention has been paid to this syndrome.

Many excellent reviews concerning diagnostic means, aetiology, and pathogenetic mechanisms have been published (44, 37, 78, 90, 112, 113), but there is still considerable disagreement regarding these aspects.

PREVIOUS INVESTIGATIONS

INCIDENCE OF COW'S MILK PROTEIN INTOLERANCE

Estimates of the incidence of CMPI in infants have produced considerably varying figures, from 0.3% to 7.5% (20, 3, 47, 40, 124) (Table 1). This variation may be due to differences in the composition of the clinical materials, to varying diagnostic criteria used for the diagnosis, and also to a variation in the incidence from one area to another. Some studies have been made retrospectively and some prospectively. To obtain optimum figures for the incidence, a prospective study is superior.

Table 1.

Incidence of CMPI

Study	Population	Incidence %	Comments
(20)	3000 Various other diagnoses	0.3	Prospective? [a]
(3)	304 Healthy infants	1.3	Prospective
(47)	1089 Healthy infants, CM[b] since birth	1.8	Prospective
(47)	669 Healthy infants, no CM[b] before 6 months of age	0.9	Prospective
(40)	787 Healthy infants	7.5	Prospective
(124)	4311 Infants born during one year	0.58	Retrospective calculated value

a) A group of patients followed in a paediatric practice, seen primarily for reasons other than CMPI. All infants referred because of suspected allergy were excluded.

b) CM = cow's milk

In Sweden (Stockholm) the incidence in 1948 was reported as 1/7500 infants (133), a figure based only on very severe reactions to cow´s milk. In 1979 the incidence in Stockholm was reported as 1/200 (124). In both, the figures for incidence of CMPI were calculated retrospectively. No prospective study on CMPI has been published in Sweden.

DIAGNOSTIC PROCEDURES

Observation of clinical effects of cow´s milk elimination and challenge

The original criteria according to Goldman et al. in 1963 (42) for making the diagnosis CMPI are based on observation of the clinical effect of elimination and the introduction of cow´s milk. Three positive challenges with return of identical symptoms were proposed neccessary. However, it is unacceptable to make repeated challenges at short intervals. Therefore according to Walker-Smith (140), most clinicians would accept one or two positive challenges as strong evidence in favour of the diagnosis. May and Block (90) proposed that the only way to make a firm diagnosis of food intolerance would be to utilize double-blind food challenges. They studied 25 infants with histories of reactions to food. Symptoms were provoked on double-blind challenge in 13. They also noted that, of 20 different food incriminated, only 4 proved responsible for all the confirmed reactions; namely, milk (7), egg (7), peanut (3), and soy (3).

Analysis of small intestinal mucosal biopsy

Several studies (38, 67, 68, 34, 70) have indicated that cow´s milk may damage the small intestinal mucosa in a variable degree, causing chronic diarrhoea and malabsorption syndrome.

In 1973 Kuitunen et al. (69) reported a study of the intestinal mucosa with both light and electron-microscopy done in 3 patients, suspected of CMPI, before and after challenge with cow´s milk. The study indicated that cow´s milk may damage the surface epithelium of the small intestinal mucosa, a damage that resembled that found in coeliac disease during gluten challenge.

The fact that cow´s milk may damage the intestinal mucosa has been used in attempts to get an objective diagnostic test for CMPI. In 1975 Shiner et al. (119) challenged 4 infants suspected of CMPI with cow´s milk (totally 150 ml). None reacted clinically, but advanced pathological changes were found in the postchallenge (within 24 h) biopsies in 2/4 patients. In consequence they proposed routine light microscopy of pre- and postchallenge jejunal biopsies as the objective criterion needed for the diagnosis of CMPI. This opinion was supported by Walker Smith et al. (141). They studied 5 infants with suspected CMPI. All got clinical symptoms on cow´s milk challenge; significant symptoms took more than 48 hours to appear (in one infant 7 days). The postchallenge small intestinal biopsy was not made before significant symptoms had appeared. They also observed patchy mucosal lesions in 5/19 specimens and stressed the importance of using a double-port capsule as the two specimens might differ.

Vitoria et al. (135) found histological changes in postchallenge biopsies in 12 out of 19 infants with CMPI. They also found similar changes after challenge with chicken meat in one infant with a chicken meat intolerance. Iyngkaran et al. (57) (studying Malaysian infants) proposed the following criteria for the diagnosis CMPI: 1) Clinical disease (diarrhoea with or without vomiting) while receiving cow´s milk protein. 2) Clinical improvement on a diet free of cow´s milk protein. 3) Normal or mildly abnormal histology of the jejunal mucosa when taken 6-8 weeks after symptoms subside. 4) Histological relapse with or without clinical relapse after re-exposure to cow´s milk protein. However, in another study Sumithran and Iyngkaran (126) found apparently no clinical justification for routine jejunal biopsy at challenge in infants suspected of CMPI. Thirty infants had significant mucosal damage after milk challenge; symptoms developed in only 22. The 8 symptom-free infants managed well on a diet containing cow´s milk and a repeat biopsy in 2 of them showed a certain recovery of the mucosal changes.

Having often found a lack of correlation between the clinical response to a cow´s milk challenge and the degree of mucosal lesion Shmerling and Rein (120) proposed an initial strict diet in infants with suspected CMPI and milk challenge after 6-8 weeks when symptom-free, without previous intestinal biopsy. They stated that only in doubtful cases would a biopsy be of help in assessing the challenge, an opinion supported by Hill et al. (53).

Ferguson and Murray (32) suggested in 1971 that counting the number of lymphocytes within the surface epithelium of the small intestine could be of value in the assessment of mucosal abnormality. In 1979 Phillips et al. (100) published a study with the aim of establishing the pattern of the intra-epithelial lymphocyte response to cow´s milk in CMPI. They found that 7 children with untreated CMPI on a milk containing diet had a significantly higher lymphocyte count than 22 control children. They also found that children on a milk-free diet, whether or not they were milk intolerant, had significantly lower counts than the control children on cow´s milk. In the 7 children with untreated CMPI, only one count approached the high levels usually found in untreated coeliac disease. A high lymphocyte count in children with untreated CMPI, but not as high as in untreated coeliac disease, was also found by Rosekrans et al. (104).

Kosnai et al. (66) studied mucosal cell kinetics and found both an increasing total number of crypt cells and an increasing number of proliferating and maturing cells in both coeliac disease and CMPI. However, they found important differences between the two diseases. The crypts were shorter and showed more vigorous mitotic activity in untreated CMPI than in coeliac disease, suggesting a more rapid enterocyte destruction in CMPI than in coeliac disease, and also a more rapid recovery of the mucosa in CMPI.

Thus, it is quite clear that cow´s milk can cause a damage of the intestinal mucosa in an infant with CMPI, a damage resembling

that found in coeliac disease during gluten challenge. In recent years, some investigations concerning intraepithelial lymphocyte counts and mucosal cell kinetics (100, 104, 66) have suggested a possibility of some differentiation between coeliac disease and CMPI. However, the value of routine light microscopy of small intestinal biopsy as a diagnostic means is still in dispute.

Immunologic investigations

Normal immunologic response to ingestion of cow's milk

Almost all children below 3 years of age have been shown to develop circulating cow's milk antibodies (84, 63). In infants milk formula fed from birth, the peak of milk IgG-antibodies was found at about 3 months of age and the peak of milk IgA antibodies at 7 months (63). Foucard (35) showed that IgE-antibodies to food allergens (egg-white, cow's milk) could be found at 3 months of age with a decrease in antibody concentration as the infants grew older. He concluded that these antibodies at low titres had no apparent relation to atopic disease. Initial breast feeding has been shown to delay the antibody response and to result in lower levels of specific milk antibodies (64, 106). The same results were shown by feeding the infants casein hydrolysate for the first three months (30). It has also been shown that prematures born after the seventh month of gestation have an onset and increase of immunoglobulin synthesis related to birth and not to gestational age (105).

Skin testing

In 1963 Goldman et al. (43) stated that positive skin tests with casein, bovine serum albumin, βLG, and αLA had little diagnostic significance as allergic children without CMPI had an even greater frequency of positive skin tests (68%) than children with CMPI (59%). Dannæus and Johansson (26) reported that 68% of the infants with immediate type of reactions to cow´s milk and 21% of the infants with delayed reactions had positive tests. They also found that the skin reactivity was greatest for βLG and greater for unboiled compared with boiled milk. Hill (53) showed that prick test was positive in 7 patients with CMPI and urticaria, but negative in 8 patients with CMPI and gastro-intestinal symptoms.

In conclusion, skin testing must be considered unreliable as a diagnostic test for CMPI.

Significance of high serum titres of milk-antibodies

It has been shown that an elevation of total IgE in cord serum could be predictive of subsequent development of atopic symptoms (61, 24).

Quantification of the levels of specific antibodies to cow´s milk proteins belonging to IgG and IgM classes has been done by several authors without significant diagnostic results (55, 110, 36). Fällström et al. (39) studied the presence of serum

antibodies, against different milk proteins, belonging to immunoglobulin classes IgE, IgM, IgG, and IgA in a group of infants with clinically manifest gastrointestinal intolerance to cow´s milk proteins. The results were compared with results from infants with gluten sensitive enteropathy, acute gastroenteritis, and one reference group. Serum antibodies against cow´s milk proteins were found in all groups of infants, and an increased antibody level of a single immunoglobulin class was considered of limited discriminating value. However, the discrimination could be increased by using combinations of increased antibody levels. Dannæus and Johansson (26) found that the clinical significance of positive RAST´s to cow´s milk was low. The concentration of milk IgE-antibodies showed a low correlation with symptoms. They even described a few RAST-negative infants who developed anaphylactoid reactions after ingestion of cow´s milk (27).

To summarize: The diagnosis of CMPI cannot be settled by a single serologic test, nor definitely by a combination of several such tests.

Local immunoglobulin production of the gut

In 18 infants with malabsorption syndrome due to CMPI, Savilathi (111) measured the numbers of immunoglobulin-containing cells in biopsy specimens and demonstrated a rise in the numbers of IgA and IgM containing cells during milk challenge in infants showing a clinical reaction to cow´s milk. Elimination of cow´s milk was followed by a normalization of the cell numbers. However, some patients without clinical reaction to cow´s milk also showed an increase in the numbers of IgA and IgM containing cells.

Few if any IgE-containing cells were seen. It was also shown that after the start of the milk challenge there was a sharp increase in IgA and IgM in faecal extracts in patients who later had clinical symptoms. On the other hand, Rosekrans et al. (104) found a markedly increased number of IgE-containing cells in the lamina propria and only a slight increase in the numbers of IgA and IgM containing cells in 8 infants with CMPI and malabsorption. An increase in mucosal IgE-containing cells as well as an increase in extracellular IgA and IgM was shown by Shiner et al. (119) during milk challenge.

In conclusion, contradictory results have been reported. Moreover, the methods used must be considered too complicated to use routinely.

Cell-mediated immune reactions

Scheinmann et al. (114) studied the value of lymphoblast transformation test in the presence of αLA and βLG in infants with CMPI. In patients with CMPI, 37.8% had a positive lymphoblast transformation test whereas 9.5% of control children had positive tests. In infants sensitized during their 1st month of life, lymphoblast transformation test was more often positive.

In 1980 Ashkenazi et al. (2) investigated the production of leukocyte-migration-inhibition factor (LIF) by peripheral lymphocytes in response to a challenge with bovine βLG in vitro.

All infants with CMPI (24) had a significant LIF production. Only 2 of 24 control patients and 2 of 10 newborns had border-values almost reaching a positive test value. Most children who had recovered from CMPI had negative assays. The authors suggested that this test could be valuable for making the diagnosis CMPI and also for determining when treatment with a cow´s milk free diet could be safely terminated. Recently Butler et al. (16) reported a decreased neutrophil chemotaxis in a group of infants with intolerance to cow´s milk and/or soy protein. A return to normal values was seen when the infants had grown tolerant to the actual foods.

To summarize, some interesting reports concerning cell mediated reactions have in recent years been published. Further studies are needed to evaluate the findings.

Complement-activation

If IgG and IgM antibodies are concerned in the pathogenesis of CMPI in some patients then the complement system is likely to be involved. In 1970 Matthews and Soothill (89) reported evidence of complement activation after milk challenge in 5/8 patients with CMPI. The main symptoms in these 5 patients were intestinal. The authors proposed that an evidence of complement activation after a milk challenge could provide a valuable test for diagnosis. Savilathi (111) was unable to demonstrate a fall in complement levels in serial blood samples taken during milk challenge in 6 infants with CMPI and clinical symptoms on challenge.

Another investigation by Dannæus (27) found decreased levels of complement not only in infants with delayed type of reactions but also in infants with immediate type of reactions, and even in some RAST-positive children who tolerated milk. From this Dannæus concluded that formation of immune complexes and activation of complement might occur as a physiological phenomenon in childhood. Yadav and Iyngkaran (148) showed that in a group of infants, with both mucosal damage and clinical symptoms on milk challenge, IgG serum levels fell in 67% of them. This fall was in many instances accompanied by a depletion of serum complement C3. However, there was also some infants without cow´s milk protein sensitive enteropathy with depletion of serum IgG and complement C3.

Thus, contradictory results have been reported. Investigations of complement levels as a diagnostic means for CMPI cannot be considered useful.

FOOD INTOLERANCE IN EXCLUSIVELY BREAST-FED INFANTS

"For most babies, breast-feeding seems to offer, in addition to the many nutritional and psychological benefits, a natural and safe means of prophylaxis for atopic disease" (107).

About half a century ago when breast-feeding was a regular custom, there was considerable interest in the occurrence of food intolerances in breast-fed infants (128, 117, 29). Nowadays, with a return to breast-feeding, there is new interest in studying

manifestations of food intolerance in totally breast-fed infants.

Using animal models, a transmission of dietary protein (bovine IgG) has been shown to occur across the placenta to the foetuses, and into the milk to the suckling rat (50, 51, 52). Matsumura et al. (88) and Kuroume et al. (72) discussed the possibility of intrauterine* and also postnatal sensitization in fully breast-fed infants with eczema and intolerance to egg, cow´s milk, and soy bean. The existence of wheat antigens in human breast-milk has been demonstrated by a modified form of passive cutaneous anaphylaxis in rat skin in 16 out of 17 randomly chosen samples of human milk (71). Gerrard (41) described 18 breast-fed infants with symptoms depending on the mothers´ intake of certain foods. The most common food responsible was cow´s milk (16 infants). Warner (142) described two infants with intolerances to food antigens transmitted through their mothers´ breast-milk. One infant developed intermittent vomiting, diarrhoea, and episodes of uncontrollable screaming from one week of age. Later on he was proved to have an intolerance to egg and cow´s milk. In the other infant, an egg intolerance was diagnosed while still on the breast by a double-blind challenge (a powder supplement was given to the mother) which objectively demonstrated that food antigen could penetrate into the breast-milk and cause disease. Recently Evans et al. (31) published the results of a double-

* In 1973 Miller et al. (94) showed that human foetuses were capable of synthetizing IgE at 11 weeks of gestation.

blind cross-over study concerning the problem of maternal diet (cow´s milk) and infantile colic in breast-fed infants. They did not find any reduction of the colic when cow´s milk was avoided in the mothers. However, they found an increase in attacks of colic together with an increasing number of different types of foods given to the mothers.

The concept that foods ingested by the mother can find their way into the breast-milk and perhaps sensitize the infant confirms what many nursing mothers have observed, namely, that foods ingested by the mother may upset the baby.

AETIOLOGICAL AND PATHOGENETIC CONSIDERATIONS

Allergenicity of cow´s milk proteins

Many studies have been devoted to the allergenicity of the cow´s milk proteins. The term allergenicity is defined as the capacity of a substance to induce allergic reactions. However, most investigators have not studied the allergenicity, but instead, the antigenicity of milk proteins. Also it should be added that neither the passive cutaneous nor other anaphylaxis tests in animals can detect the human allergy-producing antibodies.

Many studies have been done on the effect of heating cow´s milk proteins (81, 73, 102, 103, 48, 109, 95, 86, 65, 91). By

means of a complement-fixation technique it was found (81) that a casein preparation still gave unchanged reactions after heating to 110°C but had lost some of its antigenicity after heating to 120°C for one hour. The whey proteins were affected already at 60°C and lost their antigenicity rapidly at higher temperatures. Ratner et al. (102, 103) used anaphylaxis in guinea pigs to study the effect of heat on cow´s milk proteins. They claimed that α-casein was still antigenic but somewhat affected in heat-modified cow´s milk, while βLG partially and αLA completely lost their antigenicity. However, Saperstein and Anderson (109) showed that βLG and αLA were still antigenic in a variety of milk products including evaporated milk, pasteurized milk, and several infant milk formulas. The effect of heat on cow´s milk proteins studied by immuno-electrophoresis (48) showed that no components were affected at temperatures below 70°C. At 75°C the immunoglobulin component of cow´s milk proteins completely lost its ability to react with the corresponding antibody. Albumin formed a fuzzy precipitate at 85°C. After boiling the milk for 15 minutes, casein, βLG, and αLA still formed precipitates. When investigating pasteurized milk, spray-dried milk, and milk formulas αLA, βLG, and casein could be demonstrated by immunoelectrophoresis.

Heat denaturation of milk proteins has been shown to increase the digestability of the proteins with individual proteases (65). When studying tryptic hydrolysis of pure βLG, Labeyrie (73) and Monnot and Yon (95) found that the native protein was hydrolysed well, but heat-denaturation facilitated the reaction.

Several authors have shown, when challenging with purified milk proteins, that βLG is the protein that most often provokes symptoms (101, 7, 36). Davidson et al. (28) reported one child with malabsorption induced by ingestion of βLG. Bleumink and Young (7) found that of various milk proteins βLG had the highest skin reactivity. They also showed that the specific skin reactivity of βLG was considerably increased by the incorporation of lactose. This so-called browning reaction between lactose and proteins may occur during pasteurization, storage, and household handling (99), which treatment may lead to an increase of the allergenic activity of cow´s milk.

It has also been shown that digestive products (after pepsin hydrolysis) of bovine milk proteins have an antigenic specificity which differes from the parent proteins, judged by sensitization of guinea pig uterus and production of precipitins (122, 123). In 1942 Cooke (21) reported that sensitization to digestive derivatives of cow´s milk proteins existed and that in some cases delayed clinical reactions could be explained on this basis. In 1953 this hypothesis was tested (8) but could not be substantiated. Haddad et al. (46) demonstrated IgE antibodies in humans to enzymatic digests of βLG and suggested that the reactivity to these antigens might be greater than to the native protein in certain individuals. Recently Schwarts et al. (116) investigated the occurrence of specific IgE antibodies to milk proteins and new antigens generated by in vitro pepsin hydrolysis of βLG in patients with suspected

hypersensitivity to cow's milk. The findings suggested that undigested milk proteins were more reactive than digested proteins.

Absorption of macromolecules

The neonatal mammalian small intestine has a capacity to ingest macromolecules by a nonselective pinocytotic process (137, 139). Evidence exists that even the human intestine has an increased permeability to macromolecules during the neonatal period (84, 105). Implications from these studies are that the neonatal intestine may absorb antigenic material more readily than the more mature adult intestine. Contributing factors could be either a relatively large amount of protein ingested during this period, a decreased intraluminal hydrolysis, an increased leakage through an "immature" cell membrane, or a decreased intracellular protein hydrolysis. The passage of macromolecules through the intestinal mucosa could be considered a normal phenomenon mostly not giving rise to any disease states (137, 139). In individuals with an atopic diathesis this normal macromolecular absorption could be enough to sensitize and start immunological disease. This may even be more pronounced if there is an increased macromolecular transport due to alterations in the digestive processes or to a defect in the mucosal barrier.

Intraluminal factors

A decreased intraluminal digestion resulting in increased concentration of macromolecules will probably cause an enhanced

macromolecular absorption (137). This could be caused by impaired gastric or pancreatic function. The local intestinal response was enhanced when antigens were given in a bicarbonate buffer to mimic gastric anacidity (137). After pancreatic ligation Danforth and Moore (23) found an enhanced intestinal absorption of insulin in rats.

Animal studies have demonstrated that factors in maternal colostrum may enhance gammaglobulin absorption during the neonatal period (80). In a study by Weström et al. (145) neonatal piglets were fed either unprepared swine-colostrum or colostrum depleted of trypsin inhibiting activity through the addition of trypsin. A decreased absorption of all colostral proteins (IgG, albumin, colostral prealbumin) into the blood was found after feeding colostrum depleted of trypsin inhibiting activity. Whether factors in human milk can contribute to an enhanced absorption is not known. It is conceivable that such factors exist (76, 83).

The cow´s milk proteins αLA, βLG, and casein are the most commonly used substitutes for human milk proteins. None the less, there is very little information in the literature about the capacity of infants´ gastrointestinal tract to digest bovine αLA and βLG. Sparse information is available on casein (92, 54, 82). By intubation of the ileum, Hirata et al. (54) studied the capacity to digest casein in vivo, and found that, e.g., a 5 month old infant could utilize about 4 g casein in a meal. Using an in vitro model, Lindberg (82) found that infants´ duodenal juice hydrolysed on average 10 mg of casein into

trichloroacetic acid soluble peptides per ml duodenal juice per minute.

Secretory IgA

For approximately 15 years (130) it has been known that secretory IgA is the predominant immunoglobulin class present at certain mucous membranes. The IgA secreted onto the mucous membranes has been shown to be mainly produced locally by plasma cells distributed in the lamina propria of the intestinal tract and other mucosal tissues (131). Through the binding of membrane secretory component to IgA to form secretory IgA, an increased resistance to proteolytic degradation develops compared with IgA dimers lacking secretory component or with other classes of immunoglobulins (132).

The possibility that mucosal antibodies in the gut are able to exclude absorption of specific antigens has been studied and demonstrated experimentally in studies of specific antigen absorption in isolated loops of gut from immune versus non-immune animals (138). Clinically it has been shown (15) that there is a high correlation between serum milk precipitins and IgA deficiency. Taylor et al. (129) reported a prospective study of a group of children born to parents with atopic symptoms. They found that an IgA-deficiency at 3 months was associated with the development of atopy within the first year. In another study (49) it was found that 8 of 20 children with CMPI were

partially IgA-deficient, suggesting that a deficiency in IgA may be a predisposing factor for the development of CMPI.

Mucosal barrier

Alterations in the mucosal barrier is one important factor in the regulation of macromolecular transport. It has been shown in animal studies that the charge of the membranes affects the adsorption of macromolecules to cellular surfaces, thus having influence on macromolecular uptake (121).

After macromolecular ingestion by intestinal absorptive cells, most of the ingested material is broken down by lysosomal enzymes (125). Any interference with intracellular capacity to digest macromolecules could therefore result in an increased transport of macromolecules (58). A number of factors (radiation, endotoxins) can increase the lability of lysosomes causing rupture of lysosomal membranes (144).

Clinically, it has been shown (45) that serum concentrations of egg albumin after a load of egg albumin were higher in infants with diarrhoea than in control infants. The authors concluded that the most likely reason for this was an increased absorption of whole protein in patients recovering from diarrhoea, which might depend on either an increased permeability of the intestinal wall or a decreased rate of degradation within the gastrointestinal tract. Dannaeus et al. (25), studying absorption of ovalbumin

in children with malabsorption, showed that the serum determinations were influenced by antibodies (IgG) directed against ovalbumin. They concluded that the finding of only a moderate serum concentration of ovalbumin might be an effect of blocking antibodies and could not exclude the possibility of an increased absorption.

PRESENT INVESTIGATIONS

AIM OF THE INVESTIGATIONS

- To prospectively determine the incidence of CMPI in infancy in a Swedish urban community, its various manifestations, and the course of the disease during the first years of life (I).

- To evaluate routine light microscopy of pre- and postchallenge small intestinal biopsies as a routine diagnostic means in infants with suspected CMPI (II).

- To study the importance of maternal diet, particularly regarding intake of cow´s milk, in the genesis of infantile colic in breast-fed infants (III, IV).

- To study the ability of the duodenal juice, from infants with various gastrointestinal disorders, to digest the most important cow´s milk proteins (V), and to study more specifically the role of trypsin and elastase in this connection (VI).

A PROSPECTIVE STUDY OF COW'S MILK PROTEIN INTOLERANCE IN SWEDISH INFANTS (I)

Patients and methods

Malmö, a town in southern Sweden, has about 250,000 inhabitants. About 2500 infants are born each year. It has one obstetric and one children's hospital and is thus well suited for a study such as this.

During the study period, all women with a newborn infant at the obstetric hospital were informed about CMPI and about the design of the study, and were invited to participate in it.

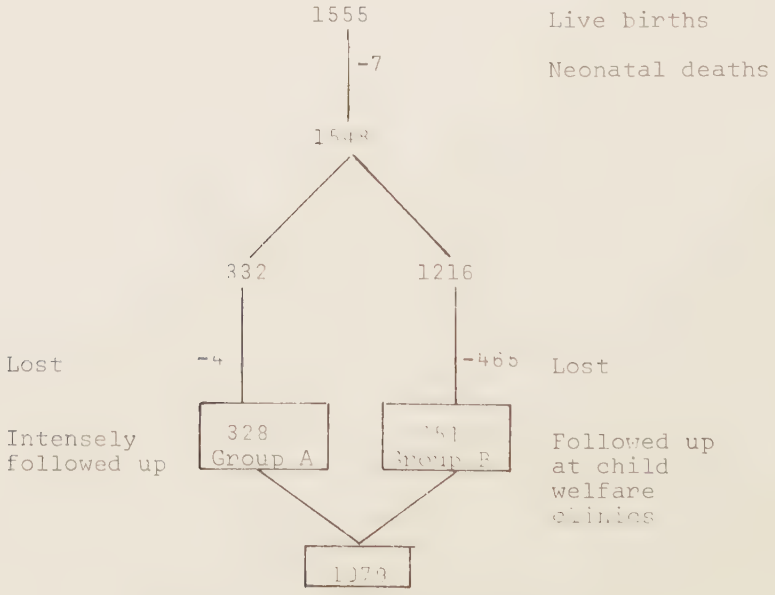

Fig 1. Survey of the number of infants.

During the period, 1555 infants were born alive (Fig 1). The parents of 332 agreed to participate, 328 fulfilled the study (Group A). From all these parents data were obtained concerning family history of allergy. The infants´ weight and height, type of feeding, and feeding problems were reported on once a month. All infants were examined at age 3, 6, and 12 months.

Of the other 1216 infants, 751 (Group B) were followed via records at well baby clinics. We examined all infants of this group with suspected CMPI who were admitted to the children´s hospital.

The diagnosis CMPI was settled when the following criteria were fulfilled:

a) the presence of symptoms on a diet containing cow´s milk;
b) the disappearance of symptoms on withdrawal of cow´s milk;
c) at least two positive milk challenges with return of identical symptoms.

Symptomatology

A total of 20 infants fulfilled the criteria. Five reacted within one hour on milk challenge and six after more than 24 h. Table 2 presents the symptoms of the 20 infants. Seven had symptoms from the gastrointestinal tract only. Five presented symptoms from the skin only, and six from both skin and gastrointestinal tract. Two had symptoms from the respiratory tract, but they also reacted from the skin and gastrointestinal tract. Seven had infantile colic.

In 3 of them colic was the only symptom at the debut, but was ignored, and they were not admitted until other symptoms occurred.

Table 2.

Symptomatology in 20 infants with CMPI

	No. of infants
Recurrent vomiting	11
Infantile colic	7
Diarrhoea	5
Eczema	7
Exanthema	7
Urticaria	4
Recurrent bronchitis	2
Slow weight gain	2

Onset of symptoms

Eleven infants had their first symptoms before 8 weeks of age. Infantile colic was the predominant symptom. Four infants had their first symptoms when fed only human milk and ten infants got their symptoms within one week after the introduction of cow´s milk. Three of them reacted at their first cow´s milk containing meal.

Other food intolerances

Twelve infants with CMPI also showed intolerances to other foods, soy protein being the most common (7/20).

Family history of allergy

A family history of allergy was found in 35% of the 328 infants. In the 20 infants with CMPI, the corresponding figure was 70%.

Development of milk tolerance

Nine infants became cow´s milk tolerant before 1 year of age. At 2 years of age, a further three had recovered. The remaining eight are now 4½ - 5 years of age. Two have developed complete food tolerance at 3-4 years of age. Three are milk tolerant but have still intolerances to other foods. Three of them have also developed a pollen and animal allergy. Three are still milk intolerant besides being intolerant to several other foods.

DO PRE- AND POSTCHALLENGE SMALL INTESTINAL BIOPSIES HELP TO DIAGNOSE COW´S MILK PROTEIN INTOLERANCE? (II)

Patients and methods

Twelve infants were studied. Symptoms (diarrhoea, vomiting, infantile colic, and/or failure to thrive) began at age 2 weeks to 6 months. Lactose intolerance was excluded. All became symptom-free and gained weight when their diet was changed to a strict cow´s milk free diet. After at least 4 weeks on this diet, they were challenged with cow´s milk. A prechallenge biopsy was taken with a hydraulic capsule at the duodeno-jejunal flexure under fluoroscopic control. The infants were then given increasing amounts

of cow´s milk. A symptomfree infant had a total of 126 ml of cow´s milk. When clinical signs appeared, the symptom-giving dose was repeated once more. A post-challenge biopsy was taken from the same region as the first one 24 hours after the challenge begun. Two or three specimens were usually taken a few centimetres apart. The specimens were examined without knowledge of clinical findings.

Clinical results

The challenge was considered positive in seven infants, two of whom had no symptoms until 2-4 days after the challenge. These seven were later rechallenged with positive results, but at one year of age, all except two tolerated a normal diet. The other five, with no clinical signs on challenge, continued without symptoms on a normal cow´s milk containing diet.

Mucosal histology

Eight infants were biopsied both before and after challenge, three before, and one after. Two or three specimens were taken at the same time at 15 out of 20 biopsy occasions. When comparing these double-specimens, 10 showed the same histological picture and 5 showed slight variations between the specimens.

There was no difference in mucosal morphology between infants with a positive challenge and those with a negative one. We could not observe any deterioration of the intestinal mucosa after the milk challenge compared with the prechallenge biopsies. In

particular there was no significant change in the epithelial cells, no increase in infiltrating inflammatory cells, no evident increase in number of mast cells, and no change in villous/ crypt ratio.

COW´S MILK PROTEINS AND INFANTILE COLIC IN BREAST-FED INFANTS (III,IV)

Patients and methods

The first study (III) observed 18 mothers with 19 breastfed infants, the second one (IV) 66 mothers with 66 breastfed infants. All infants were referred to us because of infantile colic (paroxysmal abdominal pain, persistent severe crying, abdomen distended by gas formation, and the frequent wish to suck). Except for colic all infants were healthy. The mothers were asked to completely eliminate cow´s milk from their diets for about one week initially and had both oral and written information about the diet. Then they reintroduced cow´s milk in their diets. This challenge was done twice. The infant´s behaviour (e.g. time and duration of crying, vomiting, abnormal stools, disturbed sleep) in relation to the mother´s diet was recorded on a standardized protocol.

Human milk samples (III)

Breast milk was collected from the 18 mothers on different diets. The samples were tested against laboratory supplies of rabbit antiserum to whole cow´s milk by the Ouchterlony double-diffusion technique (98).

Double-blind cross-over trial (IV)

When the colic disappeared on elimination of cow´s milk and reappeared on the mothers´ milk challenges, the mothers were asked to participate in a double-blind cross-over trial. The mothers continued their strict cow´s milk free diet during the study. Gelatinous capsules were filled with either a concentrate of cow´s milk whey proteins (200 mg) containing 67 g whey proteins per 100 g powder (V67, Semper AB, Sweden), or with potato starch at the dispensary of the hospital by a pharmacist who also coded the capsules. The capsules, which were indistinguishable, were taken by the mother according to a scheme, 3 capsules 4 times a day. The amount of whey protein in 3 capsules corresponded to the amount of whey in about 80 ml of cow´s milk. Capsules were taken on day 1 and 3. On day 5 the mother was asked to drink ½-1 glass of milk 3 times. The infant´s behaviour (e.g., time and duration of crying, vomiting, abnormal stools, disturbed sleep) during the study was recorded on a standardized protocol. The code was broken by an independent assessor at the dispensary after every fourtn patient.

Statistical method (IV)

Sequential analysis was done (13, 1) with a 10% risk of error. If the outcome was the same on both types of capsules then no information of superiority was obtained and nothing was plotted on the chart (Fig 2). If the infant reacted with colic on the

placebo-capsules, a cross was marked in the square to the right of the last entry on the chart. If the infant reacted with colic on the capsules containing cow´s milk whey proteins, a cross was marked in the square above the last entry on the chart.

Results

Immunochemical investigations (III)

Breast-milk from 13 of the 18 mothers on a diet containing cow´s milk gave distinct precipitate lines against an antiserum to whole cow´s milk (Fig 3a). Breast-milk samples from a mother who was followed up daily showed precipitates when she was on a normal diet and only on the first day of elimination of cow´s milk from the diet (Fig 3b). Breast-milk from 35 out of 49 (71%) mothers chosen at random produced precipitates.

Fig 3. Ouchterlony plates.

a) A = antiserum to cow´s milk; 1 and 2 = human milk from two mothers on a normal diet; 3 = human milk from one mother on a diet free of cow´s milk; 4 = cow´s milk.

b) A = antiserum to cow´s milk; 1 and 2 = milk from a mother while on a normal diet; 3 = milk from same mother on 1st day of a diet free of cow´s milk; 4 = milk from same mother on 2nd day of diet free of cow´s milk.

We have also found (59) precipitates between fresh human milk and rabbit antibodies to βLG by double gel diffusion technique and immunoelectrophoresis (Fig 4). The precipitates could not be produced after freezing the milk.

Fig 4. Ouchterlony plate.

A = human milk from one mother on a cow´s milk containing diet;
1 = anti-human serumalbumin; 2 = anti-cow´s milk; 3 = anti-βLG;
4 = anti-αLA.

Clinical findings (III, IV)

In the first study (III) elimination of cow´s milk to the mothers was a successful treatment, further proved by challenge, (cow´s milk to mothers) in 12 out of 19 infants. There was a family history (mother, father, siblings) in 9 of these 12 infants (75%). At age 6 months only one infant still reacted with colic on challenge, but at weaning other symptoms of cow´s milk protein intolerance (skin rash, diarrhoea, vomiting) appeared in 3 infants.

In the second study (IV) the colic disappeared in 23/66 (35%) infants after cow´s milk elimination to the mothers, and reappeared after the reintroduction of cow´s milk into the mothers´ diet. These 23 infants were further studied (Table 3). This group had a family history of allergy in 12 (52%) whereas the corresponding figure for all 66 infants was 33%.

Table 3. Survey of 23 breast-fed infants with relapse of colic on challenge with cow´s milk to the mothers.

	Debut of infantile colic	First elimination of cow´s milk to the mothers	First positive challange*	Double-blind challenge**	Negative challenge***
Range (weeks)	1-12	1-16	2-20	3-28	7-40
Mean value (weeks)	2.6	5.2	7.2	8.9	14.9

* Challenge = Cow´s milk to mother

** In 16/23 infants

*** In 18/23. 3 have a verified cow´s milk protein intolerance. 2 are only 2 and 2½ months of age.

Double-blind challenge (IV)

The double-blind challenge was done at a mean age of 8.9 weeks in 16/23 patients. Six of the sixteen mothers in the challenge had to be taken out of the study. No symptoms were registered in five infants on either the placebo or the whey protein containing capsules, not even on ½-1 glass of milk directly after the challenge with capsules. One infant (aged 3 weeks) had no reaction of either of the capsules, but did react with colic after the mother had drunk about 150 ml of cow´s milk. Five of the mothers could return to a normal diet without any problems. One mother (infant aged 3 weeks) could continue with a limited amount of milk (about 300 ml a day) without any colic in her infant.

Fig 2 gives the results of the double-blind challenge in the other 10 infants. Nine reacted with colic on the capsules containing cow´s milk whey protein and also when the mothers were drinking milk directly afterwards. No reactions were registered after the placebo capsules. One infant got colic on the placebo capsules, no colic on the whey protein capsules, but colic on the mother´s milk drinking. This mother could successively reintroduce cow´s milk in her diet within the next 2-3 weeks.

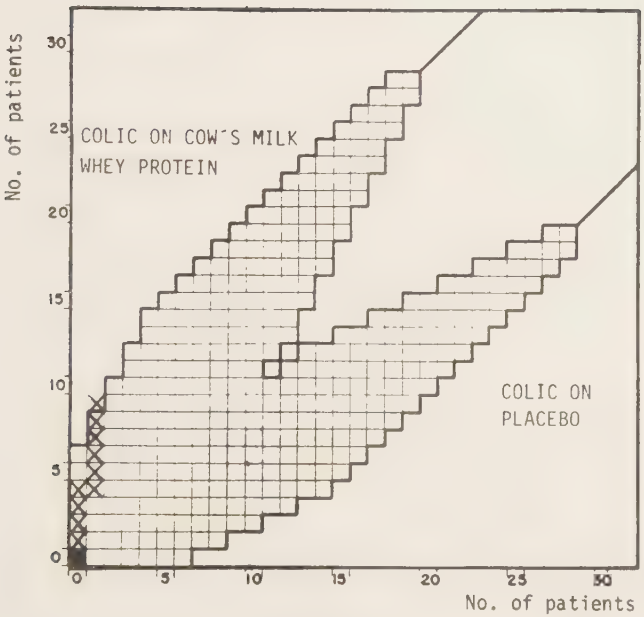

Fig 2. Double-blind cross-over trial of the effect of cow's milk whey protein to 10 mothers, on appearance of colic in their breast-fed infants.

The colic disappeared at a mean age of 14.9 weeks in 18/23 infants (Table 3). Five infants still have symptoms (October 1981). Two of them are only 2 and 2½ months of age. One infant aged 7 months has a verified (direct challenge with cow's milk) CMPI reacting with colic and vomiting on challenge at 4 months. At 6 months, she was rechallenged with cow's milk without any immediate reaction. After about one week on a milk containing diet she developed eczema. She has now returned to a cow's milk free diet and is thriving well. Two infants, aged 9 and 17 months, have multiple food intolerances including CMPI.

PARTIAL HYDROLYSIS OF COW´S MILK PROTEINS BY DUODENAL JUICE (V) AND BY HUMAN TRYPSINS AND ELASTASE (VI)

Material (V, VI)

We studied twenty infants aged 5 days to 19 months. They were admitted to the hospital because of poor weight gain and stool problems. The examination programme included small intestinal biopsy, duodenal juice aspiration, and fecal microscopy (5). Pancreatic function was assessed by determination of amylase and trypsin activities and agarose gel electrozymogram of duodenal juice (9).

The infants were divided into the following diagnostic groups: CMPI; seven infants with their symptoms disappearing after elimination of cow´s milk from the diet and return of identical symptoms at two milk challenges. Coeliac disease; five infants. Diagnosis based on a flat intestinal mucosa on a gluten-containing diet, histological and clinical improvement on a gluten-free diet, and histological and/or clinical relapse on a gluten-containing diet. Unclassified gastrointestinal disorders; six infants with failure to thrive, vomiting, and/or diarrhoea for more than 3 weeks. Normal laboratory tests. Symptoms disappeared within a few months. Pancreatic insufficiency; two infants with cystic fibrosis. Diagnosis was based on abnormal sweat tests and typical clinical course.

Duodenal juice was collected through a double lumen tube placed with the tip in the ascending part of the duodenum. The juice was collected on crushed ice and was analysed either directly or after storage at -20°C. Gastric aspirates were drawn from infants fed nasogastrically and tested for pH and proteolytic activity.

Purified human enzymes were available in the laboratory; anodal and cathodal trypsins (10), cathodal elastase (11).

Methods (V, VI)

Hydrolysis of bovine αLA. βLG, and casein was studied in purified forms (V, VI) and in standardised, pasteurised cow's milk (V, VI), two adapted formulas (casein/whey ratio 40/60) (V), and one unadapted formula (casein/whey ratio 80/20) (V). Besides a mixture was studied with the proteins in the same proportions as in cow's milk of αLA, βLG, and casein in phosphate buffer and in bovine serum (V). The substrate solution (450 µl) was incubated with 50 µl duodenal juice or enzyme solution at 37°C with continuous stirring. Aliquots (10-50 µl) were collected at different times and were mixed with an appropriate volume of ice cooled distilled water for electroimmuno-assay or with an equal volume of the anionic detergent sodium dodecyl sulphate (SDS) for SDS-polyacrylamide gel electrophoresis.

The hydrolysis was also studied after preincubation with gastric juice at 37°C for 30-60 minutes at pH 1.5-2.0 or at pH 4.5-5.0. The incubation was stopped by adding 0.4 M NaOH, raising pH to 7.5-8.0.

The hydrolysis was followed by electroimmunoassay (77) and SDS-polyacrylamide gel electrophoresis (74, 97).

Results (V, VI)

Method

When hydrolysing with duodenal juice, a comparison between electroimmunoassay and SDS-polyacrylamide gel electrophoresis showed identical results for αLA and βLG. In contrast when hydrolysing with the purified enzymes the whey proteins slowly lost the antigenicity to their corresponding antibodies. Casein gave several precipitates on electroimmunoassay (2 to 3 anodal peaks), making it impossible to determine its hydrolysis with this method.

Clinical material (V)

No hydrolysis occurred in the two patients with pancreatic insufficiency. The other three groups showed no significant differences.

The capacity to hydrolyse αLA and βLG was not correlated with age in the actual age group. The rate of hydrolysis of the various proteins was slower with a crude substrate solution as in cow´s milk or infant formulas. To study the influence of the composition of the substrate solution on the rate of hydrolysis, αLA, βLG, and casein were mixed in phosphate buffer or in bovine serum. $T_{\frac{1}{2}}$ (= time required to hydrolyse 50% of the protein) for αLA was

5 minutes when present as a single protein or together with βLG and casein in a phosphate buffer solution. $T_{\frac{1}{2}}$ for αLA in bovine serum was 12 minutes and in cow´s milk 37 minutes. Corresponding results for βLG were 8, 10, 19, and 25 minutes, respectively.

On average one ml of duodenal juice hydrolysed 0.84 mg of purified αLA, 0.94 mg of purified βLG, and 29.3 mg of casein per minute (Table 4). Table 4 also demonstrates that the ability of duodenal juice to hydrolyse the proteins in purified form exceeded the ability to hydrolyse them in cow´s milk by about 30 times for αLA, by 10 times for βLG, and twice for casein.

Table 4. Capacity of infants´ duodenal juice to hydrolyse α-lactalbumin, β-lactoglobulin, and casein (mg protein/ml duodenal juice/minute). Mean values ± SEM (N).

Substrate / sis	Purified						Cow´s milk					
	α-lactalbumin [a]		β-lactoglobulin [a]		casein [b]		α-lactalbumin [a]		β-lactoglobulin [a]		casein [b]	
milk n rance	0.76	0.18 (7)	1.16	0.20 (6)	14.0, 33.6		0.03	0.01 (5)	0.15	0.05 (5)	13.6, 27.2	
e	0.78	0.41 (3)	0.82	0.20 (3)	35.6, 26.4		0.02	0.01 (3)	0.11	0.03 (3)	6.0, 15.0	
sifed intestinal er	1.03	0.28 (4)	0.70	0.16 (4)	34.4, 32.0		0.03	0.01 (4)	0.09	0.04 (4)	16.8, 18.2	
y	0.84	0.14 (14)	0.94	0.12 (13)	29.3	3.3 (6)	0.03	0.01 (12)	0.12	0.03 (12)	16.1	4.2

sured by electroimmunoassay and SDS-polyacrylamide gel electrophoresis.

sured by SDS-polyacrylamide gel electrophoresis. Individual observations are given.

Preincubation with gastric aspirate at pH 1.5 showed a considerable hydrolysis of the various proteins. At pH 4.5 there was no detectable hydrolysis of the various proteins in purified form or in cow's milk after incubation for 60 minutes. The subsequent incubation with duodenal juice gave the same hydrolytic rates as without gastric preincubation.

Hydrolysis by human trypsins and elastase (VI)

Table 5 gives the results. The rate of hydrolysis of the various proteins by cathodal elastase exceeded that by anodal or cathodal trypsin. Casein was hydrolysed more efficiently than αLA and βLG.

Table 5. Partial hydrolysis of purified α-lactalbumin, β-lactoglobulin, and casein by human trypsins and elastase (mg protein/mg enzyme/minute).

	α-lactalbumin		β-lactoglobulin		Casein
	Method A *	Method B **	Method A *	Method B **	Method B **
Anodal trypsin (2 experiments)	n.d. ***	0.14	n.d.	0.49	-
	n.d.	0.25	n.d.	0.60	3.67
Cathodal trypsin (3 experiments)	n.d.	0.15	0.15	0.59	11.2
	0.19	0.08	0.18	0.18	-
	0.10	0.12	0.13	0.77	11.8
Cathodal elastase (3 experiments)	0.40	2.25	1.33	3.0	47.0
	0.60	1.80	1.83	3.8	21.7
	0.60	2.70	1.17	4.3	36.0

* Hydrolysis measured by electroimmunoassay

** Hydrolysis measured by SDS-polyacrylamide gel electrophoresis

*** n.d. = no detectable hydrolysis

**** Hydrolysis of casein was measured by SDS-polyacrylamide gel electrophoresis, only

Hydrolysis of standardised cow´s milk by cathodal trypsin or cathodal elastase showed that the ability to hydrolyse the proteins in purified form exceeded the ability to hydrolyse them in cow´s milk by about 10-15 times for βLG and by about 3 times for casein. There was no detectable hydrolysis of αLA in cow´s milk.

GENERAL DISCUSSION

The incidence of CMPI was determined at 1.9% in Swedish infants. However, in the group most intensely studied (group A), it was 3.7%. Parents with allergy problems of their own are more interested in taking part in a prospective study. Therefore there is most probably an inevitable selection in group A. In this group 35% of the infants had a family history of allergy, but this figure does not differ much from that reported by Kjellman (62) in a Swedish unselected population (about 30%). Moreover group A probably includes infants with mild forms of CMPI not otherwise diagnosed.

Recently, Verkasalo et al. (134) reported a changing pattern of CMPI in Finland, when comparing occurrence and clinical course in the 60s and mid-70s. Infants treated for CMPI before 1974 had a longer period of symptoms before diagnosis, greater retardation of growth and more severe intestinal damage than infants treated after 1974. This was believed to reflect mainly the increasing awareness of the disease in both laymen and paediatricians.

The symptoms of the infants in our study were relatively mild. The nature of the study made the symptomatic period short. For instance, only two infants presented with symptoms of malabsorption, i.e. failure to thrive. The pattern of symptoms at the debut agreed with previous studies (20, 3, 19, 37, 40, 53). Gerrard et al. (40) found that about 40% of the infants with CMPI developed

their symptoms within one week after their first exposure to cow´s milk. Our corresponding figure was 70%. Four infants had symptoms already when fed only human milk and three at their first cow´s milk containing meal. This supports the hypothesis that sensitization to cow´s milk proteins can occur in utero (88, 72) or via the breast-milk (117, 29).

Sixty per cent of the infants with CMPI showed adverse reactions to foods other than cow´s milk. It is important to bear in mind this increased tendency towards associated food intolerances when introducing new foods in the diet of an infant with CMPI.

Gerrard et al. (40) found that 27% had lost their sensitivity to cow´s milk before 2 years of age. Clein (19) reported as many as 95%. Our corresponding figure was 60%. Of the other 40% (8 infants) 4 infants must be considered true allergic according to definition. They all had eczema as one symptom at the debut of their CMPI. All 4 have multiple allergies (to food, inhalants, animals) but 3 of them are now at age 5 years cow´s milk tolerant. A further 2 are still intolerant to several foods including cow´s milk.

The diagnosis CMPI in the infants of the prospective study was based on clinical observation of the effects of elimination and introduction of cow´s milk. In view of the results reported in

the literature when studying the intestinal mucosa before and after milk challenge (119, 141, 57, 135) we tried to use this procedure diagnostically in 12 infants; challenge was considered positive clinically in 7. The study revealed the patchy nature of the mucosal lesions previously noticed by Walker-Smith et al. (141). In contrast to previous investigators (119, 141, 57, 135) no deterioration of the intestinal mucosa was found after milk challenge compared with the prechallenge biopsies. Possible reasons for this could be a smaller challenge-dose of cow´s milk or that the infants had less severe initial symptoms. It has been shown (70) that in CMPI the intestinal mucosa is damaged to a varying degree. Thus the milk dose might be critical for the appearance of mucosal abnormalities. It is well known that the clinical reactions can be severe after giving cow´s milk to these infants (e.g., severe diarrhoea requiring intravenous fluids). Therefore we considered a further increase in the amount of milk unjustified. It may be debatable whether the infants with a clinically positive challenge really had CMPI, or were the reactions due to some intercurrent illness. However, all reacted with similar symptoms on at least two milk challenges and lactose intolerance was excluded. Therefore we were convinced that the infants had CMPI.

Analysis of intestinal disaccharidases or dipeptidases may be a more sensitive test of mucosal damage. Walker-Smith et al. (141) found that analyses of intestinal disaccharidases in pre- and postchallenge biopsies were a helpful guide to mucosal relapse. However, these authors did the postchallenge biopsy after more

than 48 hours on cow´s milk and the biopsies showed a morphological deterioration. It is known that the intestinal disaccharidase and dipeptidase activities are significantly correlated to morphological findings (4). In addition, the results of enzyme analyses can be difficult to estimate as the mucosal lesion in this disorder may be patchy.

It can be concluded that a postchallenge biopsy at 24 hours without any histological deterioration does not exclude the possibility of CMPI and that the clinical result of milk challenge does not necessarily run parallel with the morphological results, judged by routine methods.

However, in CMPI as in other malabsorption syndromes, a small intestinal biopsy is the only way to determine the existence of a mucosal lesion (5). Moreover, in doubtful cases, it is an invaluable means for following the effect of elimination and the reintroduction of cow´s milk over a longer period.

In the prospective study (I) 4 infants had symptoms of CMPI already when fed only breast-milk, 3 of them had infantile colic. This led us to study further the problem of infantile colic in breast-fed infants.

Several authors (19, 42, 40) have described colic as a common symptom of CMPI. A recent double-blind study of infantile colic in formula-fed infants (85) showed that 53% of the infants reacted to both cow´s milk and soy formula, but were free of symptoms on a formula containing casein hydrolysate. At about 3 months of age a challenge with a cow´s milk formula gave symptoms of infantile colic in 36% of the infants.

The extended experience on infantile colic in breast-fed infants (IV) showed that the colic was in 1/3 of the infants related to the mothers´ cow´s milk consumption. This was further proved by the double-blind cross-over trial. Our results contradict those of Evans et al. (31). They studied 20 breast-fed infants with infantile colic. According to our results the colic could be expected to depend on cow´s milk in only 6 or 7 of these infants. The most important reason that Evans et al. did not find any effect of cow´s milk elimination may be their choice of soy milk as placebo. A placebo substance should be a substance of no importance as an allergen in infancy. Soy protein is an important allergen in infancy (113) and has been reported as antigenic as cow´s milk proteins (30).

The factor(s) in cow´s milk appearing in human milk and producing colic in the infants has not yet been identified. We continue this work.

Evidence exists that even the human intestine has an increased permeability to macromolecules during the neonatal period (84, 105). One contributing factor to this could be a decreased intraluminal hydrolysis. This has also been proposed as one possible cause (37) of the relatively high incidence of CMPI in infancy.

We investigated in vitro the hydrolysis of the bovine proteins αLA, βLG, and casein. The initial steps of the digestion by gastric and duodenal juice (V) and by the purified human trypsins and elastase (VI) were followed by two different methods. In electro-immunoassay (76) the basic principle of quantitative estimate of proteins by precipitation with the corresponding antibody is used. Theoretically, parts of a peptide chain can be split off without interfering with its antigenic sites. The other method, SDS-polyacrylamide gel electrophoresis, separates the proteins according to the molecular mass of their polypeptide chains (116, 141) and amount of unsplit polypeptide is measured. When hydrolysing αLA and βLG by duodenal juice a comparison between the two methods showed identical results (V). With human trypsins and elastase the two methods showed different results, i.e. αLA and βLG slowly lost the antigenicity to their corresponding antibodies (VI).

Human trypsin has been found to be well developed already at one month of age (79) but a continuing development has been found (12) of cathodal elastase during the first two years of life. No correlation was found between a low cathodal elastase content and the diagnosis CMPI (12). However, the infants were studied when

they already had a manifest CMPI and not at the time when they were sensitized to cow´s milk proteins.

In our study the rate of hydrolysis of the three proteins by cathodal elastase exceeded that by the trypsins. This difference may well be explained by the narrow substrate specificity of trypsin (acting upon peptide links formed from the carboxyl group of either arginine or lysine) and the broader specificity of elastase (acting upon peptide links with alifatic amino acids).

The duodenal juice from the infants with CMPI hydrolysed αLA, βLG, and casein as efficiently as the duodenal juice from the other infants. This does not support the theory that a defect proteolytic activity of the duodenal juice contributes to the pathogenesis of CMPI. However, as discussed above, the infants were studied when they already had a manifest CMPI, and we have no information on proteolytic capacity of their duodenal juice when they were sensitized to the proteins.

During infancy gastric acidity is low, especially so in the newborn infant (93). Peptic activity decreases rapidly as the pH rises from 2 to 7, becoming very weak at pH 4, and completely inhibited by pH 7 (147). A human milk meal resulted in a sharp rise in gastric pH to 5-6, which was maintained for about 60 minutes (87, 17). When feeding cow´s milk formula, a pH of 5-6 was maintained for more than 2 hours (17). Mason (87) also found that in the majority of cases very little human milk was left in the stomach when the pH had fallen to values optimum for peptic digestion.

The hydrolysis of αLA and βLG occurred at a considerably slower rate when present in crude form as in cow´s milk or in adapted and unadapted formula, than when in purifed form. The reason for this is obscure. A mixture of αLA, βLG, and casein in phosphate buffer did not affect the rate of hydrolysis by duodenal juice of the individual proteins. However, when the three proteins were mixed in preheated bovine serum, the hydrolysis occurred at a slower rate but not as slow as in cow´s milk. Thus it seems that some protease inhibiting factor(s) is present in cow´s milk, in formulas, and probably in bovine serum (146).

Nutritional (14, 6) and antigenic (14) effects of feeding adapted (casein/whey = 40/60) or unadapted (casein/whey = 80/20) formula have been studied. Nutritionally, no or only small differences were found. In the well-baby population studied by Buckley and Dees (14), no increased antigenicity, demonstrated by tanned cell hemagglutinins, positive skin tests, or occurrence of precipitins to cow´s milk was found in the infants fed adapted formula. There was no difference in the occurrence of wheezing, urticaria, or eczema between infants fed adapted or unadapted formula. Of a total of 488 infants, 151 remained at the end of the study. Those who dropped out for reasons suggestive of CMPI did not return for further investigations. Therefore no diagnoses of CMPI could be settled. Of the 151 infants remaining in the study, one infant of each feeding group was felt to have feeding difficulties (colic, frequent splitting up) during the first months of life.

We found that the rate of the initial hydrolysis of casein exceeded that of αLA and βLG both when hydrolysed by duodenal juice and by the trypsins and elastase. This difference was even greater when hydrolysing the three proteins contained in cow´s milk. Considering this, together with the fact that bovine whey proteins have been reported as important allergens in infancy (101, 7, 36), the use of adapted infant formulas instead of unadapted may, in spite of the findings of Buckley and Dees (14), be questioned from an allergic point of view.

GENERAL SUMMARY

- The incidence of CMPI in Swedish infants was 1.9% (20/1079) in a prospectively studied group. Four infants had their first symptoms when fed only human milk and ten infants got symptoms within one week after the introduction of cow´s milk. Twelve infants with CMPI also showed intolerances to other foods. Nine infants became cow´s milk tolerant before age 1 year. At age 4½-5 years, three are still milk intolerant.

- Small intestinal biopsy was studied with routine light microscopy before and 24 h after the start of milk challenge in 12 infants. In 7, the challenge was considered positive when judged clinically. No difference in mucosal morphology was found between infants with a positive challenge and those with a negative one. No deterioration of the intestinal mucosa was observed after the milk challenge compared with the prechallenge biopsies. When comparing double-specimens taken at the same time at 15 biopsy occasions, 10 showed the same histological picture and 5 showed slight variations between the specimens.

- Infantile colic in breast-fed infants has been shown in 1/3 of the infants to be related to the mothers´ cow´s milk consumption. This was further proved by a double-blind cross-over trial.

- Partial hydrolysis of αLA, βLG, and casein by duodenal juice, by human anodal and cathodal trypsin, and cathodal elastase was

studied in vitro. Duodenal juice from infants with CMPI hydrolysed the three bovine proteins as efficiently as duodenal juice from infants with coeliac disease or unclassified gastrointestinal disorder. The rate of hydrolysis of casein exceeded that of αLA and βLG both when hydrolysed by duodenal juice and by the trypsins or elastase. The hydrolysis of αLA and βLG occurred at a considerably slower rate when present in crude form as in cow's milk than in purified form. The rate of hydrolysis of the various proteins by cathodal elastase exceeded that by anodal or cathodal trypsin.

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I

A PROSPECTIVE STUDY OF COW'S MILK PROTEIN INTOLERANCE IN SWEDISH INFANTS

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ABSTRACT. Jakobsson, I. and Lindberg, T. (Department of Paediatrics, Malmö General Hospital, Malmö, Sweden). A prospective study of cow's milk protein intolerance in Swedish infants. Acta Paediatr Scand, 68: 853, 1979.—1 079 of 1 548 newborn infants were followed during their first year. 328 were prospectively contacted once a month. 751 were followed up at child welfare clinics. Altogether 20 were diagnosed as being cow's milk protein intolerant (1.9%). Symptoms from the gastrointestinal tract and the skin predominated. Only 2 had respiratory symptoms. Ten had their symtpoms within one week after the introduction of cow's milk, 3 of them at their first cow's milk-containing meal. A further 4 already had symptoms when fed only human milk. The others (6 infants) showed symptoms after more than one week on a cow's milk containing diet. Before 2 years of age, 13 had recovered. Twelve of the cow's milk protein intolerant infants also showed adverse reactions to other foods, soy-protein intolerance being the most common (7 infants). A family history of allergy was found in 35% (116) of the 328 infants and in 70% (14) of those with cow's milk protein intolerance.

KEY WORDS: Cow's milk, cow's milk protein intolerance, food intolerance

Adverse effects from foods are on record from ancient times. Hippocrates observed that cow's milk could cause gastric upset and urticaria (4), but food hypersensitivity was rarely reported before von Pirquet introduced the concept of allergy in 1906 (32). Since 1966, the term allergy has been used mostly to describe IgE-mediated hypersensitivity reactions (18, 21). It is still not known how many hypersensitivity reactions to cow's milk proteins are mediated. Therefore we prefer the term cow's milk protein intolerance, meaning any adverse reaction, whether of immediate or delayed type, to cow's milk proteins, giving symptoms in the gastrointestinal tract, the skin, or the respiratory tract.

Estimates of the incidence of cow's milk protein intolerance in infants have produced considerably varying figures, from 0.3% to 7.5% (1, 5, 6, 13, 15, 31). This variation may be due to differences in the composition of the clinical materials, to varying diagnostic criteria used for the diagnosis, and also to a variation in the incidence from one area to another.

This study determined the incidence of cow's milk protein intolerance in infancy in a Swedish urban community, its various manifestations, and the course of the disease during the first years of life.

PATIENTS AND METHODS

Malmö, a town in southern Sweden, has about 250 000 inhabitants. About 2 500 infants are born each year. It has one obstetric and one children's hospital. During the period of this study, 45% of the infants in Malmö were entirely breastfed at 3 months, and 20% at 6 months (L. Righard, M.D., Head of Child Welfare Clinics in Malmö, personal communication). From 9 February to 24 March 1976 and 11 October 1976 to 17 April 1977, all women with a newborn infant at the obstetric hospital were informed by one of us (I. J.) about cow's milk protein intolerance. They received both oral and written information about the design of the study and were invited to participate in it.

During these periods, 1 555 infants were born alive; 7 died neonatally. Of the other 1 548, the parents of 332 agreed to participate. Four failed to fulfil the study (Fig. 1). This group of 328 comprise *Group A*. Of the other 1 216, 751 (*Group B*) were followed up via records at child welfare clinics attended by physicians from the children's hospital or practitioner paediatricians. The physicians and the nurses at the child welfare clinics were informed

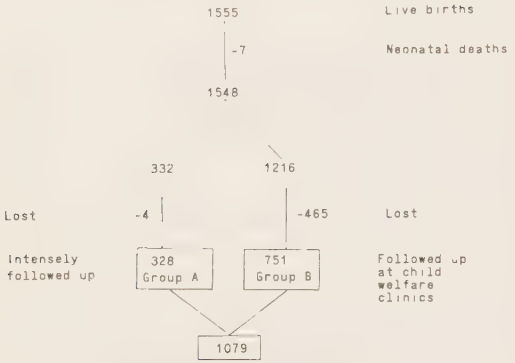

Fig. 1. Survey of the number of infants.

about the study. Altogether 1 079 infants were followed up. Of the other 465 (see Fig. 1) one-third had left the city, the records of one-third could not be found, probably because of a change of surname, and of one-third because of moving several times from one district to another within the city.

Follow-up examinations

Group A. Data were obtained from all parents concerning family history of allergy (eczema, urticaria, asthma, hay fever). Once a month, the parents gave, on a standardized form, data concerning each infant's height and weight (obtained at the child welfare clinics) and type of feeding. Questions concerning problems were also answered (infantile colic, diarrhoea, vomiting, bronchitis, rhinitis, exanthema). The parents could telephone any day to discuss problems.

All infants were examined by one of us (I. J.) at age 3, 6, and 12 months. Those with symptoms that might be ascribed to allergy were examined more frequently.

Group B. Infants suspected of cow's milk protein intolerance were admitted to the children's hospital and examined by us. We studied the records of the 751 infants at child welfare clinics with special regard to type of feeding, gain in weight and height, feeding difficulties, infantile colic, eczema, or recurrent bronchitis during their first year of life. Finally, the hospital records of those infants admitted because of any problem were also studied.

Managements of infants suspected of cow's milk protein intolerance

Cow's milk protein intolerance was suspected in any infant with one or more of the following symptoms: vomiting and/or diarrhoea not due to any other demonstrable cause, infantile colic not disappearing after advice on feeding technique or treatment with dimethylpolysiloxane (Minifom®), eczema, urticaria and exanthemas without known relation to other foods, and recurrent bronchitis.

The infant was given a diet free from cow's milk—if possible human milk, otherwise a soy-based formula. Those not tolerating soy-protein were given a formula based on casein hydrolysate (Nutramigen®, Mead Johnson). Infants with symptoms when fed human milk initially continued at the breast with the mother on a cow's milk free diet.

If the symptoms disappeared on a cow's milk protein free diet, the infant was challenged with whole cow's milk. The challenges were performed after a period of cow's milk withdrawal varying from 4 to 20 weeks (mean 8.4 weeks). (In one infant, it was performed after 2 weeks with a negative result.) Gradually increasing amounts of whole cow's milk were given every third hour, beginning with 1 ml and finishing with 50 ml. The challenge was completed when a distinct positive reaction was seen. At a suspected positive reaction, the last dose was repeated once. A symptom-free infant had a total of 126 ml cow's milk.

We made no challenges to diagnose food intolerances other than cow's milk protein intolerance. However, the parents spontaneously often performed challenges, and the reactions were carefully recorded.

Diagnostic criteria

The diagnosis of cow's milk protein intolerance was based on: (*a*) the presence of symptoms on a diet containing cow's milk; (*b*) the disappearance of symptoms on withdrawal of cow's milk; (*c*) at least two positive milk challenges with return of identical symptoms.

Lactose intolerance was excluded by a careful history; by the infant tolerating human milk; and, in doubtful cases, by a blood glucose rise of ≥ 1.1 mmol/l and no symptoms following a lactose tolerance test (dose 2 g/kg bodyweight).

RESULTS

Incidence

A total of 20 infants (11 boys and 9 girls) fulfilled the criteria given above and were diagnosed as cow's milk protein intolerant (Table 1). Five reacted within one hour after challenge and six after more than 24 h (24–96 h). Cow's milk proteins were eliminated from the diet in a further 30 because of suspected symp-

Table 1. *Incidence of cow's milk protein intolerance*

For explanation of groups A and B, see Fig. 1

	No. of infants	%
Group A (n=328)	12	3.7
Group B (n=751)	8	1.1
Total (n=1 079)	20	1.9

Table 2. *Symptomatology and course of the disease in 20 infants with cow's milk protein intolerance*

Patient	Introduction of cow's milk age (weeks)	Onset of symptoms age (weeks)	Elimination of cow's milk age (weeks)	Symptoms[a]	Positive milk challenges age (weeks)	Tolerant to cow's milk age (weeks)	Intolerances to other foods
FA ♂	20	20	28	*D*, B, E	32, 60, 96	>104	Orange, tomato, soy protein
PS ♂	8	44	51	E	72, 104	>104	Orange, egg, fish
VL ♀[c]	16	3	3	*C*, D, V, Ex, E	8, 24, 72	>104	Wheat, soy protein
FT ♂[c]	Never	6	16	E	20, 40, 72	>104	Orange, egg, wheat, nuts, soy protein
AS ♀	10	24	24	*C*, V, U, Ex, E	28, 52	>104	Fish, soy protein
CA ♀[b]	16	16	20	*V*, E	32, 40, 72, 104	>104	Orange, tomato
TM ♀	24	24	44	D	60, 80	>104	–
MG ♂[b]	18	18	24	*V*, *U*, Ex, E	40, 48	>104	Egg, soy protein
AN ♂[c]	20	4	4	Ex	8, 12	20	–
DL ♂	1/2	26	28	U, Ex	36, 40	48	Several fruit juices
EI ♀	16	16	16	*V*, *Ex*, D	20, 28	44	–
MH ♂	3	20	28	E	32, 40	48	Orange, rosehip, egg, fish
AK ♀[c]	8	4	4	*C*, V	8, 16	28	–
TJ ♀	5	6	12	C, V	16, 24	36	Rosehip, soy protein
MD ♂[b]	8	8	8	V	16, 40	60	–
ME ♂	2	6	9	V, W	12, 16	40	Soy protein
AR ♂	3	3	9	*C*, *V*, *D*, W	16, 20	28	–
PH ♀	2	4	24	C, V	32, 40	48	–
DS ♀	8	8	15	C, U, Ex	20, 40	56	–
DE ♂	2	2	8	*E*, B	12, 24	52	Orange

[a] V=vomiting, D=diarrhoea, C=infantile colic, U=urticaria, Ex=exanthema, E=eczema, B=bronchitis, W=slow weight gain. Italics=first symptom(s).
[b] Symptoms at first cow's milk containing meal.
[c] First symptoms when fed only human milk.

toms. In 7, the elimination had no effect. In 23, the symptoms disappeared and did not return when the infants were challenged with cow's milk. They continued on a cow's milk-based formula without any symptoms. They were followed for at least 2 months after the challenge, 12 up to one year of age.

Symptoms

Table 2 presents symptoms and course of the disease in the infants with cow's milk protein intolerance. Seven infants had symptoms from the gastrointestinal tract only; in 2 combined with a slow weight gain. One had diarrhoea as the predominant symptom; a 6-month-old girl, tolerating human milk, got diarrhoea and abdominal pain on cow's milk on several challenges. A lactose tolerance test was normal. She had normal stools on a cow's milk free diet and no signs of intestinal infection. Secondary lactose intolerance could thus be excluded. Seven infants had infantile colic. In 3 of them, colic was the only symptom at the debut, but was ignored, and the infants were not admitted until other symptoms occurred. Five infants presented symptoms from the skin only, and 6 from both the skin and gastrointestinal tract. Two had symptoms from the respiratory tract, but they also reacted from the skin and intestinal tract. Four of the infants had symptoms when fed only human milk. The symptoms disappeared with the mothers on a cow's milk free diet and returned with the mother on a normal diet. Identical symptoms appeared on direct challenge with whole cow's milk.

Table 3. *Onset of symptoms in relation to the introduction of cow's milk in infants with cow's milk protein intolerance*

	No. of infants[a]
<1 week	10[b]
1–4 weeks	2
>4 weeks	4

[a] Four infants had their first symptoms when fed only human milk.
[b] Three had their first symptoms at first cow's milk containing meal.

All 5 infants reacting within one hour after challenge vomited, 2 also had urticaria and exanthema, one colic and diarrhoea. Of the 6 infants reacting after more than 24 h after challenge 3 had eczema, 1 diarrhoea, 2 diarrhoea combined with colic and vomiting.

Onset of symptoms

Eleven infants had their first symptoms before 8 weeks, 8 between 12 and 26 weeks, and one boy at 44 weeks of age (Table 2). The latter had been fed cow's milk since 8 weeks of age, but got cow's milk dependent eczema at 44 weeks of age. Infantile colic was the predominant symptom in the infants reacting before 8 weeks of age (6/11).

Table 3 shows that 10 infants got their symptoms with one week after the introduction of cow's milk. Three reacted on their first cow's milk containing meal.

Infants who became cow's milk protein intolerant were entirely breastfed as long as the other infants (13 weeks *vs.* 12 weeks (mean value)).

Duration of the intolerance

Nine infants became cow's milk tolerant before 1 year of age, 3 of them before 6 months (Table 2). At 2 years of age, a further 3 had recovered. Eight infants aged 2–3 years are still intolerant to cow's milk; 7 of them have multiple food intolerances and eczema.

Associated food intolerances

Twelve infants with cow's milk protein intolerance also showed intolerance to other foods (soy, egg, fish, wheat, tomato, orange, nuts). Seven were intolerant to soy protein, which was the most common food intolerance associated with cow's milk protein intolerance. Ten infants had adverse reactions to several types of foods (see Table 2).

Intolerances to foods other than cow's milk

Adverse reactions to foods other than cow's milk (e.g. egg, fish, wheat, tomato, orange, strawberry) were reported in 26% (85) of the Group A infants. Many of these reactions were diffuse exanthemas after eating some kind of fruit. The reactions had occurred at least twice before we considered them as food dependent. These intolerance periods were usually brief—a few months.

Allergic heredity

A family history of allergy (mother, father, and/or sibling) was found in 35% (116) of the 328 infants in Group A. In the 20 infants with milk protein intolerance the corresponding figure was 70% (14).

DISCUSSION

The diagnosis of cow's milk intolerance is still clinically based; so far, there is no laboratory test which can distinguish one individual with this condition from others (3, 7, 8, 10, 12, 19, 26, 28).

The diagnosis must therefore be based on clinical observation of the effects of elimination, and introduction of cow's milk. The original criteria for making the diagnosis of cow's milk protein intolerance, according to Goldman (14), with three positive milk challenges, is often impossible to fulfil. It is often unacceptable to make repeated milk challenges at short intervals. This procedure also probably leads to fewer diagnoses of the disorder, as the

infants can have become tolerant before the third challenge. Therefore most clinicians now would accept one or two positive challenges as strong evidence in favour of the diagnosis (33); we used two positive challenges as a diagnostic criterion.

It is important to exclude lactose intolerance as a cause of the infant's symptoms. Mostly, this is easily done by a careful history. In only one infant in our study had a lactose tolerance test to be made. The results of lactose tolerance tests could be of value, but must be interpreted with caution as up to 25% might be false-positives (2, 17, 23). It is also evident that lactose intolerance might accompany cow's milk protein intolerance (16, 25). Moreover, it has been reported that commercial lactose preparations might contain small amounts of cow's milk proteins (30).

Previous studies on the incidence of cow's milk protein intolerance gave figures varying from 0.3 to 7.5%. The incidence 0.3% is given by Collins-Williams (6) after excluding all patients with major allergies and does not represent a normal population. A retrospective study by Bachman & Dees (1) found that 1% of 403 infants had cow's milk protein intolerance. Recently Stintzing & Zetterström retrospectively calculated an incidence of 0.58% (31). The incidence in two other prospectively studied groups of infants is reported to be 1.5% (15) and 7.5% (13). The overall incidence in our study was 1.9%, but in the group most intensely studied (Group A) it was 3.7%. Naturally, parents with allergy problems of their own are more interested in taking part in a study such as this. Therefore there is probably an inevitable selection in Group A with a 35% family history of allergy, but this figure does not differ much from that reported by Kjellman (30%) in a Swedish unselected population (22). Moreover, Group A probably includes infants with mild forms of cow's milk protein intolerance not otherwise diagnosed.

The symptomatology at the debut agrees with previous studies (1, 5, 6, 11, 13). Gerrard et al. (13) found that about 40% of the infants with cow's milk protein intolerance developed their symptoms within one week after their first exposure to cow's milk. In our study, the corresponding figure is 14/20 (70%). Four had symptoms already when fed only human milk and three at their first cow's milk containing meal. This supports the hypothesis that cow's milk protein sensitization can occur *in utero* (27) or via human milk (9, 20, 29). A further 4 infants of Group A with severe infantile colic when fed only human milk were free of symptoms when their mothers had a cow's milk free diet (20). They were challenged several times with positive results by giving the mothers a normal diet. However, when challenged directly with whole cow's milk at age 3–4 months, they had no symptoms and thus did not fulfil our diagnostic criteria.

The frequency of soy-protein intolerance in cow's milk protein intolerant infants in our study (35%) agrees with other reports (11), but is higher than that of Kuitinen et al. (24), who studied infants with malabsorption syndrome because of cow's milk intolerance.

26% of Group A had adverse reactions to foods other than cow's milk. Of the infants with cow's milk protein intolerance, the corresponding figure was 60%. It is important to bear in mind this increased tendency towards associated food intolerances, also reported by others (11, 13, 24), when introducing new foods in the diet for the cow's milk protein intolerant infant.

Gerrard et al. (13) found that 27% had lost their sensitivity to cow's milk before 2 years of age. Clein (5) reports as many as 95% before 2 years. Our corresponding figure is 60%. The future prospects of those who are cow's milk protein intolerant in infancy are obscure. No infant of our study has so far been followed for more than 3 years. According to Clein (5), 80% of infants intolerant to cow's milk develop other allergic manifestations before they reach the age of puberty.

In conclusion, the incidence of cow's milk protein intolerance in infancy in a Swedish urban community was 20 out of *c.* 1 000. In 14

of the 20, the onset of symptoms occurred within one week after first exposure to cow's milk. Thirteen of the 20 recovered from the intolerance before 2 years of age.

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II

DO PRE- AND POSTCHALLENGE SMALL INTESTINAL BIOPSIES HELP TO DIAGNOSE COW'S MILK PROTEIN INTOLERANCE?

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ABSTRACT. Berg, N. O., Jakobsson, I. and Lindberg, T. (Department of Paediatrics, Malmö General Hospital, and Department of Pathology, University of Lund, Sweden). Do pre- and postchallenge small intestinal biopsies help to diagnose cow's milk protein intolerance? Acta Paediatr Scand, 68: 657, 1979.—Twelve infants suspected of cow's milk protein intolerance were challenged with cow's milk after at least one month of cow's milk-free diet. The challenge was clinically positive in seven. Small intestinal biopsies were taken with a multipurpose capsule both pre- and postchallenge (at 24 h) in eight, prechallenge in three, and postchallenge in one. Two or three biopsy specimens were taken at the same time in 15 of the 20 biopsy occasions. The morphology of the mucosa could vary from normal to slight damage at the same biopsy occasion. No difference was found in morphology judged by light microscopy between pre- and postchallenged biopsies. Light microscopy of small intestinal biopsies taken before and at 24 h after milk challenge seems of doubtful value as a routine diagnostic means in cow's milk protein intolerance.

KEY WORDS: Cow's milk protein intolerance, small intestine, small intestinal biopsy

The study of the clinical response to elimination and reintroduction of cow's milk has long been the only way to diagnose cow's milk protein intolerance (5). The small intestinal mucosa is damaged in variable degree in this disease and shows no characteristic changes (4, 10, 12, 17, 19, 21). Histological, ultrastructural, and immunological changes of small intestinal mucosa after challenge with cow's milk have been reported (6, 7, 9, 11, 12, 14, 15, 17, 18, 20, 21). The value of routine light microscopy of pre- and postchallenge (at 24 h) small intestinal biopsies for the diagnosis of cow's milk protein intolerance has been discussed (3, 6, 15, 16, 17, 18, 20). The patchiness of the mucosal lesions (17, 20, 21) perhaps limits the value of this method.

To evaluate this procedure as a routine diagnostic means, we studied the morphology in multiple biopsies from twelve infants with suspected cow's milk protein intolerance. Eight were biopsied both before and after challenge with cow's milk, three before, and one after. The results are reported here.

PATIENTS AND METHODS

Twelve infants were studied, seven had an heredity to allergic diseases. Symptoms (diarrhoea, vomiting, infantile colic, and/or failure to thrive) began at age 2 weeks to 6 months. In eight, the symptoms appeared within one month on a cow's milk containing formula. Three developed symptoms on breast milk and were free of symptoms with the mothers on a cow's milk-free diet (8). Lactose intolerance was excluded.

All became symptomfree and gained weight when their diet was changed to a strict cow's milk-free diet (soy formula, Nutramigen® (Mead Johnson), or cow's milk-free breast milk). After at least 4 weeks on this diet, they were challenged with cow's milk. After premedication with chloralhydrate, a prechallenge biopsy was taken with a hydraulic capsule (13) at the duodeno-jejunal flexure under fluoroscopic control.

The infants were then given increasing amounts of cow's milk (1 ml to 50 ml) every third hour and two 50 ml doses of cow's milk 12 hours and 2 hours before biopsy. A symptomfree infant had a total of 126 ml of cow's milk. When clinical signs appeared, the symptom-giving dose was repeated once more. The postchallenge biopsy was taken from the same region as the first one 24 hours after the challenge began. Two or three specimens were usually taken a few centimeters apart.

The biopsy specimens were oriented on a millipore filter and fixed in 4% formol solution with short after-fixation in Bouin's solution. They were then examined and photographed in a dissecting microscope. After embedding in paraffin or plastic the biopsy was serially cut into 5–6 μm

Table 1. *Clinical and morphological results of milk challenge in cow's milk protein intolerance*

Patient	Cow's milk introduced age (d)	Onset of symptoms age (d)	Cow's milk-free diet age (d)	Milk challenge age (d)	Total amounts of milk (ml)	Clinical results	Morphological results: Prechallenge Villi	Prechallenge Histology[a]	Postchallenge Villi	Postchallenge Histology[a]
P. O. ♀	7	24	75	150	126	Positive[b] diarrhoea	(*a*) Leaves+ridges (*b*) Leaves	SL SL	(*a*) Leaves+ridges (*b*) Leaves+ridges	SL N?
T. J. ♀	30	30	45	120	11	Positive vomiting, diarrhoea	(*a*) Leaves+ridges (*b*) Ridges	SL SL	(*a*) Leaves+ridges (*b*) Leaves+ridges	SL SL
L. Å. ♀	30	60	120	150	126	Positive[b] vomiting, diarrhoea	(*a*) Leaves (*b*) Leaves	SL SL	Leaves+ridges	SL
F. A. ♂	180	180	210	240	126	Positive diarrhoea	(*a*) Leaves+ridges (*b*) Leaves	SL SL	(*a*) Ridges (*b*) Ridges (*c*) Leaves+ridges	N? SL SL
A. J. ♀	2	60	120	180	126	Positive colic, diarrhoea	(*a*) Ridges (*b*) Ridges (*c*) Ridges	N? N SL	– –	– –
C. A. ♀	115	115	150	235	11	Positive vomiting	(*a*) Ridges (*b*) Not evaluable	SL SL	– –	– –
O. T.[c] ♂	–	30	30	90	11	Positive colic, diarrhoea	–	–	(*a*) Finger+Leaves (*b*) Leaves	N? N?
M. G.[c] ♂	–	14	45	90	126	Negative	Ridges	SL	Leaves+ridges	SL
A. K.[c] ♀	–	45	45	180	126	Negative	Finger+leaves	N?	(*a*) Leaves (*b*) Leaves	SL SL
F. E. ♂	30	60	75	150	126	Negative	(*a*) Not evaluable (*b*) Ridges	SL SL	(*a*) Ridges (*b*) Ridges	SL SL
J. P. ♂	45	60	75	180	126	Negative	(*a*) Leaves (*b*) Leaves+ridges	N? SL	Ridges	SL
J. F. ♂	120	130	150	225	126	Negative	(*a*) Leaves (*b*) Leaves+ridges	N? SL	–	–

[a] N=normal; N?=uncertain, slight cell infiltration, otherwise normal, epithelial cells normal. SL=slight damage; inter epithelial lymphocytes, distinct foci of plasma cells and/or leucocytes (mostly eosinophils), slight elongation of the crypts.
[b] 2–4 days after challenge.
[c] Breastfed infants with severe colic, disappearing with the mother on a cow's milk-free diet.

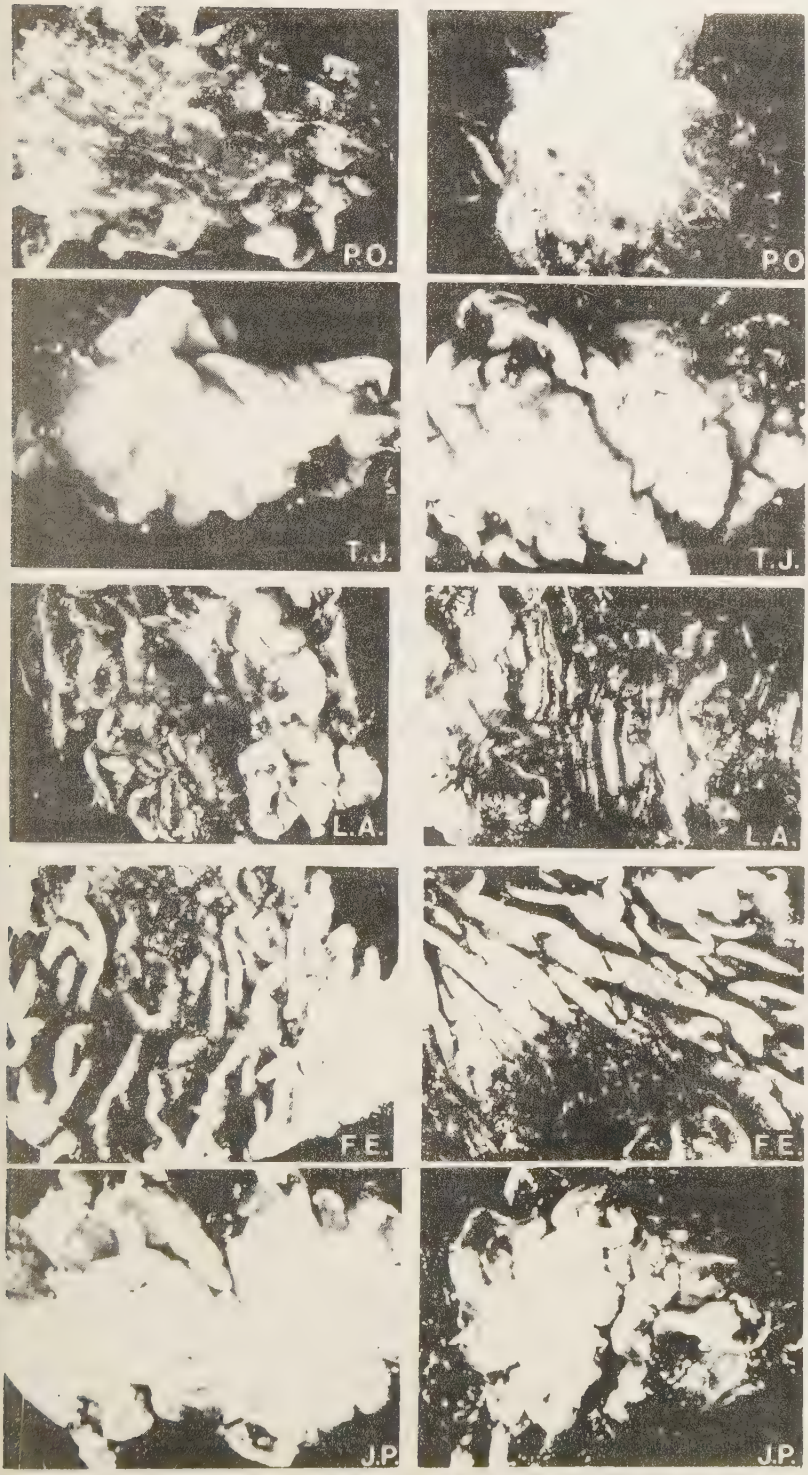
sections and 1–2 μm sections respectively. The sections were stained with Htxl-Eosin, PAS according to McManus, and Giemsa stain for mast cells. The best oriented central cores of the specimens were used for assessment. The specimens were examined without knowledge of clinical findings.

Infants with symptoms on challenge had a cow's milk-free diet. Symptomfree infants continued to have a cow's milk containing formula at home and were controlled regularly. If no symptoms reappeared within twelve months, the challenge was judged negative. All the infants were followed for at least one year after the challenge.

RESULTS

Table 1 gives some clinical data and the clinical and morphological results of the challenge. The challenge was considered clinically positive in seven infants, two of whom had no symptoms until 2–4 days after the challenge. A cow's milk-free formula was successfully reintroduced in all seven. They were later rechallenged with positive results, but at one year of age, all except two tolerated a normal

Fig. 1. Appearance of intestinal mucosa in the dissecting microscope before (to the left) and after (to the right) milk challenge in five children. For P. O., T. J. and L. Å. the challenge was clinically positive but for F. E. and J. P. the challenge was negative. Slender or broader leaves and slightly increased ridging is seen for P. O. and L. Å., but also for J. P.


P.O.
P.O.
T.J.
T.J.
L.A.
L.A.
F.E.
F.E.
J.P.
J.P.

diet. These two are still, at two years of age, on a cow's milk-free diet because of eczema. The other five, with no clinical signs on challenge, continued on normal cow's milk containing diet. They had no symptoms and had gained weight normally one, two, and twelve months after the challenge.

Eight infants were biopsied both before and after the challenge, three before, and one after. Two or three specimens were taken at the same time at 15 out of the 20 biopsy occasions. When comparing these double-specimens, ten showed the same histological picture and five showed slight variations between the specimens (Table 1). There was no difference in mucosal morphology between infants with a positive challenge and those with a negative one (Fig. 1 and Table 1). We could not observe any deterioration of the intestinal mucosa after the milk challenge compared with the prechallenged biopsies. In particular, there was no significant change in the epithelial cells, no increase in infiltrating inflammatory cells, no evident decrease in number of mast cells, and no change in villous/crypt ratio.

DISCUSSION

There is a need for an objective and rapid method for diagnosing cow's milk protein intolerance. Therefore the reports (6, 7, 15, 17) on even major (6, 7, 17) changes of morphology in intestinal biopsies 24 h after challenge with cow's milk were promising.

The present study found only slight morphological abnormalities of the intestinal mucosa. Thus in no case were distinct convolutions seen in the dissecting microscope, and in well orientated sections, the villous/crypt height ratio was always more than 1 : 1.

The study also revealed the patchy nature of the mucosal lesions (17, 20, 21). The specimens in 5 of 15 biopsy occasions showed a histological variation from normal to slight damage, irrespective of whether the biopsy was taken pre- or postchallenge and whether the challenge was clinically positive or negative.

We saw no definite changes in dissecting and light microscopy of the small intestinal mucosa at 24 hours after the challenge began compared with the prechallenge morphology, irrespective of whether the challenge was clinically positive or negative. From the infants with a positive clinical response, 13 mucosal specimens were taken on 6 pre- and 11 at 5 postchallenge biopsy occasions. On the whole, there were no more abnormalities in the postchallenge mucosal specimens. These findings are in contrast to those of Shiner et al. (15), Sumithran & Iyngkaran (17), and Iyngkaran et al. (6, 7). The reason for this is perhaps that these authors gave a larger challenge-dose of milk and that the infants had more severe symptoms initially. In cow's milk protein intolerance, there is a varying degree in the tolerance of the mucosa to the cow's milk proteins (12), and the milk dose might be critical for the appearance of mucosal abnormalities. It is well known that the reactions can be severe after giving cow's milk to these infants (e.g. severe diarrhoea requiring intravenous fluids because of dehydration (21)), and we considered that a further increase in the amount of milk was unjustified. In our opinion the symptoms and signs presented by the infants in this report are representative for infants with cow's milk protein intolerance in this part of the world. The infants were not treated earlier and for a longer time before challenge than in the previous studies (6, 7, 15, 17).

Finally, it is debatable whether the infants with a clinically positive challenge really had cow's milk protein intolerance, and whether instead, the reactions were due to some intercurrent illness. However, all reacted with similar symptoms at at least two more cow's milk challenges. Therefore we are convinced that the infants had cow's milk protein intolerance.

From this study, it can be concluded that a postchallenge biopsy at 24 h without any histological deterioration does not exclude the possibility of cow's milk protein intolerance, and that the clinical result of milk challenge

does not necessarily run parallel with the morphological results judged by routine methods. This design of diagnostic procedure seems of doubtful value.

Analyses of intestinal disaccharides or dipeptidases may be a more sensitive test of mucosal damage. Walker-Smith et al. (21) found that analyses of intestinal disaccharidases in pre- and postchallenge biopsies were a helpful guide to mucosal relapse. However, these authors did the postchallenge biopsy after more than 48 h on cow's milk and the biopsies showed a significant morphological deterioration. It is known that the intestinal disaccharidase and dipeptidase activities are significantly correlated to morphological findings (2). In addition, the results of enzyme analyses can be difficult to estimate as the mucosal lesion, both pre- and postchallenge, can be patchy in this disorder.

We emphasize that in cow's milk protein intolerance, as in other malabsorption syndromes, a small intestinal biopsy is the only way to determine the existence of a mucosal lesion (1). Moreover, in doubtful cases, it is an invaluable means for following the effect of elimination and reintroduction of cow's milk over a longer period (11, 21).

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COW'S MILK AS A CAUSE OF INFANTILE COLIC IN BREAST-FED INFANTS

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Summary 18 mothers of 19 breast-fed infants with infantile colic were put on a diet free of cow's-milk protein. The colic disappeared promptly from 13; in 12, it reappeared on at least two further indirect challenges (in the form of a diet containing cow's milk to the mother). Most infants became symptom-free at age 2 to 4 months; at 4 months, only 4 reacted with colic when the mother took cow's milk. 5 infants were directly challenged with cow's milk; 4 reacted promptly with colic. Other signs of intolerance to cow's-milk protein developed in 3 infants during weaning. The treatment of infantile colic in breast-fed infants by a diet free of cow's milk for the mother appears worthwhile.

Introduction

INFANTILE colic is common. Suggestions regarding its cause include intestinal hyperperistalsis, disturbances in the mother-child relation, and food intolerance.[1-3]

Colic is a well-known sympton of intolerance to cow's-milk protein.[4-8] Since infantile colic also affects breast-fed infants, it may be caused by cow's-milk proteins transmitted from mother to infant via breast milk. We therefore gave a diet free of cow's milk to some mothers whose breast-fed infants had infantile colic. We report the clinical findings and some preliminary results of an immunochemical investigation. A preliminary report of the study has been published.[9]

Patients and Methods

We observed 18 mothers with 19 breast-fed infants (8 boys and 11 girls), 11 of them first children. All were delivered normally at term, and 12 had a family history (mother, father, or sibling) of allergic disease. The colic started 1–4 weeks after birth.

All infants in the study were routinely examined because of infantile colic (paroxysmal abdominal pain and severe crying). Crying, measured in hours per day, was recorded. All mothers were informed that colic is a common symptom of allergy to cow's milk in infancy and that we

wished to find out whether cow's milk taken by a mother could cause colic in her breast-fed infant. The mothers were asked to eliminate cow's milk (including food containing cow's milk) from their diets, for about a week before cow's milk was reintroduced into the diet. Elimination of cow's milk from the diet and its reintroduction were repeated at least twice. The infant's behaviour in relation to the mother's diet was recorded.

Breast milk was collected from the 18 mothers when they were on their different diets. Breast milk was collected daily from one mother both when she was on a normal diet and when she eliminated cow's milk from her diet. Milk samples were also collected from 49 randomly chosen mothers. The samples were analysed directly or stored at −20°C until analysis.

The human milk samples were tested against laboratory supplies of rabbit antiserum to whole cow's milk by the Ouchterlony double-diffusion technique,[10] with 0·8% agarose (Miles Seravac, Maidenhead, England) in 0·075 mol/l barbital-sodium-barbital, pH 8·6, containing 2 mmol/l calcium lactate.

Results

Case Reports

Patient 1.—This was a boy with no family history of allergic disease who was fed only human milk from birth. Colic started at 4 weeks (fig. 1). At 6 weeks, cow's milk was eliminated from the mother's diet and the colic almost completely disappeared within a day, but promptly recurred when the mother returned to a normal diet. At age 13 weeks, the boy was directly challenged with 11 ml cow's milk. He promptly reacted with colic and diarrhœa. An intestinal biopsy at the duodeno-jejunal flexure 24 h after the challenge showed normal mucosa, except for slight cell infiltration, the significance of which was uncertain. He was then given a soy-based formula, on which he thrived and gained weight. At 6 months, he did not react to a direct rechallenge with cow's milk and has since (for 2 years) been on a normal diet without any symptoms.

Patient 2.—This was a girl with a family history of allergic diseases who was fed only human milk from birth. Colic started at age 3–4 weeks (fig. 2). When cow's milk was eliminated from the mother's diet the colic promptly disappeared. Several challenges (a glass of milk to the mother) resulted in symptoms of colic within half an hour of a breast feed. At 3½ months, while still on breast milk, she did not react when challenged, and the mother was able to take cow's milk again. When she was being weaned at 6–7 months, she had diarrhœa and skin rashes when given cow's milk and soy-based formula. The symptoms disappeared when she was given a formula containing hydrolysed casein ('Nutramigen', Mead Johnson). Since then, challenge with cow's milk on four occasions has

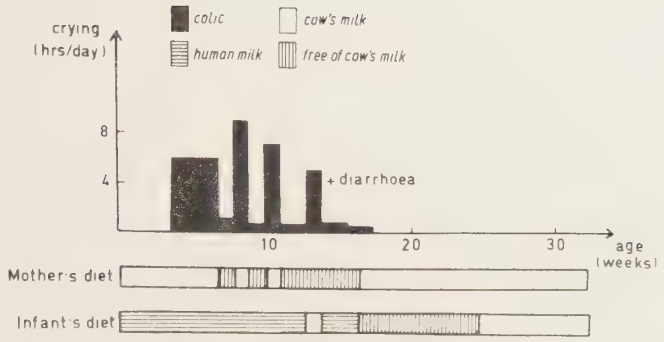

Fig. 1.—Symptoms in relation to diet (patient 1).

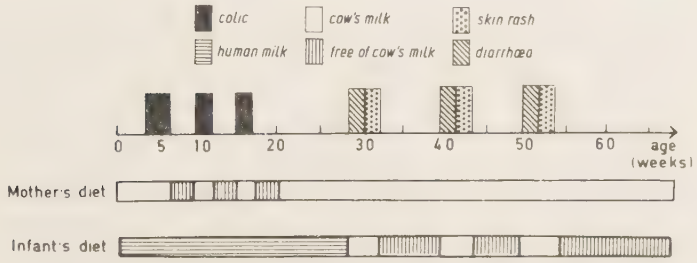

Fig. 2.—Symptoms in relation to diet (patient 2).

produced skin and gastrointestinal reactions each time. Now, aged 18 months, she still does not tolerate cow's-milk proteins. At age 10 months, she also became intolerant to wheat, but not to oats. A small-intestinal biopsy after several months on a diet rich in wheat showed slight damage of the mucosa but not a picture of cœliac disease. She has also become allergic to pollen (conjunctivitis and rhinitis).

Patient 3.—She is the younger sister of patient 2, and from birth was given human milk only. At 3 weeks, she had colic. When cow's milk was eliminated from the mother's diet the colic disappeared. She was challenged several times and reacted positively each time, but by age 4 months, she could tolerate limited amounts of food containing cow's-milk proteins in her mother's diet. However, a skin rash and facial œdema developed when the mother ate an egg. She is also allergic to pollen (conjunctivitis and rhinitis). At 4½ months, a direct challenge with 0·5 ml cow's milk gave her colic and skin rash in 15 min.

Patient 4.—This was a boy with no family history of allergic diseases, who was given human milk from birth. Colic started at age 2–3 weeks. When cow's milk was eliminated from the mother's diet a week later, the colic disappeared, but he reacted positively to challenges at 5 and 6 weeks. At 3 months, reintroduction of cow's milk into the mother's diet did

not result in symptoms in the infant. He was fed human milk until the age of 5 months but since then (for 1½ years) he has taken a normal diet containing cow's milk without getting symptoms.

Summary of Clinical Findings

When cow's milk was eliminated from the mothers' diet, the colic disappeared within 1–2 days in 13 of the infants (see table). In 1 infant, the effect was uncertain. In 5, elimination of cow's milk from the mother's diet had no effect; however, the mother of one of these 5 infants did not adhere strictly to the instructions—she stopped drinking milk but continued to eat foods containing milk.

Symptoms reappeared within hours of reintroduction

CLINICAL FINDINGS

—	No. of patients
A. Elimination of cow's milk-proteins from mothers' diet	
Disappearance of the colic	13
Uncertain effect	1
No effect	5
*B. Challenge**	
1. Initially	
Relapse of the colic	12
Uncertain effect	2
No effect	5
2. At age 4 mos	
Relapse of the colic	4
No effect	8†

*Challenge=cow's milk to mother.
†1 of these infants (patient 2) had symptoms (skin rash and diarrhœa) when being weaned at age 6–7 months and at 18 months still does not tolerate cow's-milk proteins.

of cow's milk into the mothers' diet, in 12 out of 19 infants (see table). New challenges produced reactions in all 12. By age 4 months, only 4 had colic on challenge; in 8, the symptoms disappeared between 2 and 4 months (see table). At age 6 months, 3 tolerated cow's-milk proteins, but the fourth still reacted with colic. Of the 5 infants who were directly challenged with cow's milk at age 4 months, 4 reacted with colic.

At weaning, other symptoms of cow's-milk protein intolerance (skin rash, vomiting, diarrhœa) appeared in 3. The families of 9 of the 12 infants (11 mothers) had a history of allergic disease.

Immunochemical Investigations

Breast-milk from 13 of the 18 mothers on a diet containing cow's milk gave distinct precipitate lines against cow's-milk-protein antiserum (fig. 3a). Of the 13 samples, 9 came from the 11 mothers whose infants reacted to cow's milk and 4 from the other 7 mothers. Breast-milk samples from the mother who was followed up daily showed precipitates when she was on a normal diet and only on the first day of elimination of cow's milk from the diet (fig. 3b). Breast-milk from 35 out of 49 (71%) mothers chosen at random produced precipitates.

Discussion

It is generally recognised that substances are passed from mother to infant via breast-milk. Many mothers avoid foods that they know from experience will cause reactions such as vomiting, diarrhœa, colic, or skin rash in their breast-fed infants. However, there are few published reports giving details of such substances, especially of macromolecules. In 1966, Gamo et al.[11] found cow's-milk proteins in human milk by the use of passive cutaneous anaphylaxis. In 1975, Matsumura et al.[12] showed egg-antigen in breast-milk, cord blood, amniotic fluid, and meconium by the use of passive cutaneous anaphylaxis.

The exclusion of cow's-milk proteins from the diet also results in the elimination of foods such as milk chocolate. Chocolate taken by the mother is commonly said to cause diarrhœa in the breast-fed infant. Recently, Resman et al.[13] showed that a few hours after a mother has eaten chocolate, the breast-milk contains fairly large amounts of theobromine, a substance known to relax smooth muscle and to stimulate the central nervous system. However, they found no adverse effects in the infants.

Our clinical findings strongly suggest that some factor or factors in cow's milk produces colic in infants. The finding of precipitates by the Ouchterlony technique suggests that a macromolecular substance eaten by the mother enters her breast-milk. There is increasing evidence that the normal intestine is permeable to macromolecules in quantities that might be antigenic or biologically active.[14] The nature of the precipitates is unknown.

Why only some infants get colic is not known. The cause of the colic is probably multifactorial. Several of

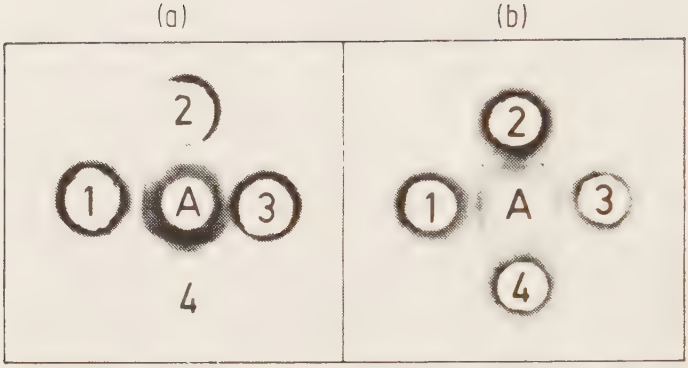

Fig. 3.—Ouchterlony plates

(a) A=antiserum to cow's milk; 1 and 2=human milk from two mothers on a normal diet; 3=human milk from one mother on a diet free of cow's milk; 4=cow's milk.

(b) A=antiserum to cow's milk; 1 and 2=milk from mother while on a cow's-milk-containing diet; 3=milk from same mother on 1st day of a diet free of cow's milk; 4=milk from same mother on 2nd day of diet free of cow's milk.

the mothers of the infants with colic were heavy milk drinkers (> 1 litre/day). Recently, specific secretory IgA antibodies against cow's-milk proteins were shown in human milk.[15] Whether these antibodies were present in sufficient amounts or were qualitatively able to carry out their presumed biological function in binding cow's-milk proteins is open to speculation. Predisposing factors might also play a role, since a family history of allergic disease was common.

The disappearance of the colic may have been due to the elimination of cow's milk per se or to psychological factors. The following points support the view that the cow's milk in the mother's diet caused the colic in her breast-fed infant—the effect of challenge and elimination of cow's milk was shown at least twice; 4 out of 5 infants directly challenged with cow's milk during the time that they were sensitive to cow's milk in the mother's diet reacted with colic; other signs of intolerance to cow's-milk protein developed in 3 infants when they were being weaned.

We conclude that infantile colic in breast-fed infants can be related to cow's-milk consumption by the mother and we suggest that the treatment for infantile colic in breast-fed infants is a diet free of cow's milk for the mother.

We thank Mrs B. Benediktsson and Mrs M. Mansson for technical assistance.

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IV

COW´S MILK PROTEINS CAUSE INFANTILE COLIC IN BREAST-FED INFANTS

A double-blind cross-over study

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ABSTRACT. 66 mothers of 66 breast-fed infants with infantile colic were put on a diet free from cow´s milk. The colic disappeared in 35 infants; it reappeared on at least two challenges (cow´s milk to mother) in 23 infants (35%). A double-blind cross-over trial with cow´s milk whey protein was done in 16 of these 23 mothers and infants. Sequential analysis showed a high correlation between infantile colic in breast-fed infants and their mothers´ cow´s milk protein consumption. We suggest a diet free of cow´s milk for the mothers as a first trial of treatment of infantile colic in breast-fed infants.

KEY WORDS: Infantile colic, human milk, cow´s milk proteins, milk-free diet

It has been recognized for about 50 years that substances can pass from mother to infant via the breast-milk and cause adverse reactions (e.g., allergic) in the infant[1-4].

Some years ago we reported[5], on clinical grounds, that infantile colic in breast-fed infants could be related to the mothers´ cow´s milk consumption. This conclusion was not supported by the recent studies of Evans et al[6].

This paper reports our extended experience on this topic and the results of a double-blind cross-over trial. The results support our previous findings.

PATIENTS AND METHODS

We observed 66 mothers with 66 breast-fed infants. All infants were referred to us because of infantile colic (paroxysmal abdominal pain, persistent severe crying, abdomen distended by gas formation, and the wish to suck often) either from the nurses at the well-baby clinics in the city of Malmö or from the hospital out-patient clinic. All infants were examined by one of us (I.J.) on at least three occasions. Except for the colic all were healthy. The mothers were asked to eliminate cow´s milk totally from their diets for about one week initially, and had both oral and written information about the diet. Then they reintroduced cow´s milk in their diets. This challenge was done

twice. The infant´s behaviour (e.g., time and duration of crying, vomiting, abnormal stools, disturbed sleep) in relation to the mother´s diet was recorded on a standardized protocol. When the colic disappeared on elimination of cow´s milk and reappeared on the mothers´ milk challenges, the mothers were asked to participate in a double-blind cross-over trial. The mothers continuing with a diet free of cow´s milk had a supplement of calcium.

Double-blind cross-over trial

A randomised double-blind cross-over study was done. The mothers continued their strict cow´s milk free diet during the study. Gelatinous capsules were filled, with either a concentrate of cow´s milk whey proteins (200 mg) containing 67 g whey proteins per 100 g powder* (V67, Semper AB, Sweden), or with potato starch, at the dispensary of the hospital by a pharmacist who also coded the capsules. The capsules, which were indistinguishable, were taken by the mothers according to a scheme, 3 capsules 4 times a day. The amount of whey protein in 3 capsules corresponded to the amount of whey in about 80 ml of cow´s milk. Capsules were taken on day 1 and day 3. After further two days the mother was asked to drink ½-1 glass of milk 3 times. The infant´s behaviour (e.g., time and duration of crying, vomiting, abnormal stools, disturbed sleep) during the study was recorded on a standardized protocol. The code was broken by an independent assessor at the dispensary after every fourth patient.

* The other 33 g consisted of fat (1.5 g), carbohydrate (19 g), minerals and electrolytes.

The study was approved by the Ethical Committee of the University of Lund.

Statistical method

Sequential analysis was done[7, 8] with a 10% risk of error (Fig 1.). If the outcome was the same on both types of capsules, then no information of superiority was obtained and nothing was plotted on the chart. If the infant reacted with colic on the placebo-capsules, a cross was marked in the square to the right of the last entry on the chart. If the infant reacted with colic on the capsules containing cow´s milk whey proteins, a cross was marked in the square above the last entry on the chart.

RESULTS

The elimination of cow´s milk from the mothers´ diet resulted in the colic disappearance within 1-3 days in 35 of the 66 infants (53%) (Table 1). Symptoms reappeared within 1 to 8 hours after reintroduction of cow´s milk into the mothers´ diet in 23 (35%) of the 35 infants. The mothers of the remaining 12 infants could return to a normal cow´s milk containing diet without their infants showing any symptoms.

The 23 infants (11 boys and 12 girls) with relapse of colic after the mothers had drunk cow´s milk constituted the group further studied (Table 2). In this group there was a family

Table 1. Results of cow's milk elimination and challenge* in 66 breast-fed infants with infantile colic

		No. of patients
A.	Elimination of cow's milk proteins from mothers' diet	66
	Disappearance of the colic	35
B	Milk challenge*	35
	Relapse of the colic	23

* challenge = cow's milk to mother. Done twice.

Table 2. Survey of 23 breast-fed infants with relapse of colic on challenge with cow's milk to the mothers

	Debut of infantile colic	First elimination of cow's milk to the mothers	First positive challenge*	Double-blind challenge**	Nega chal
Range (weeks)	1 - 12	1 - 16	2 - 20	3 - 28	7
Mean value (weeks)	2.6	5.2	7.2	8.9	14

* Challenge = Cow's milk to mother

** In 16/23 infants

*** In 18/23. 3 have a verified cow's milk protein intolerance. 2 are only 2 and 2½ months of age.

history of allergy in 12 (52%), whereas corresponding figure for all 66 was 33%. Mean value for the debut of the colic was 2.6 weeks. Corresponding value for all 66 infants was 2.5.

The double-blind challenge was done at a mean age of 8.9 weeks in 16/23 patients. Seven did not participate for several reasons: One infant reacted with a fulminant urticaria after the mother had drunk cow´s milk; she reacted in the same way when hospitalized and was given pooled human milk. Therefore, we could not for ethical reasons continue with the challenges. Four mothers did not agree to participate in the double-blind challenge. Two mothers did not have enough breast-milk; an adapted cow´s milk based formula resulted in intensive colic in one infant and colic in combination with vomiting in the other. The infants were both given a soy protein based formula with success, and were at age 4 and 7 months, respectively, challenged with cow´s milk without any symptoms.

Six of the sixteen mothers and infants who participated in the double-blind challenge had to be taken out of the study. No symptoms were registered in five infants (aged 7, 8, 10, 11 and 15 weeks) on either the placebo or the whey protein containing capsules, not even on ½-1 glass of milk directly after the challenge with capsules. One infant (age 3 weeks) had no reaction on either of the capsules, but did react with colic after the mother had drunk about 150 ml of cow´s milk. Five of the mothers could return to a normal diet without any problems. One mother (infant aged 3 weeks) could continue with a limited amount of milk (about 300 ml a day) without any colic in her infant.

Figure 1 gives the results of the double-blind challenge in the other 10 infants. Nine reacted with colic on the capsules containing cow´s milk whey protein and also when the mothers were drinking milk directly afterwards. No reactions were registered after the placebo capsules. One infant got colic on the placebo capsules, no colic on the whey protein capsules, but colic on the mothers´ milk drinking. This mother could successively reintroduce cow´s milk in her diet within the next 2-3 weeks.

The colic disappeared at a mean age of 14.9 weeks in 18/23 infants. Five infants still have symptoms. Two of them are only 2 and 2½ months of age. One infant aged 7 months has a verified (direct challenge with cow´s milk) cow´s milk protein intolerance reacting with colic and vomiting on challenge at age 4 months. At 6 months she was rechallenged with cow´s milk without any immediate reaction. After about one week on a milk containing diet she developed eczema. She has now returned to a cow´s milk free diet and is thriving well. Two infants, aged 9 and 17 months have multiple food intolerance including cow´s milk protein intolerance.

DISCUSSION

The present results support the hypothesis that infantile colic can be a symptom of food intolerance even in the breast-fed infant[4-6, 9]. In formula fed infants colic is a well-known symptom of intolerance to cow´s milk protein[10,11]. Our extended experience on colic in breast-fed infants presented here suggests

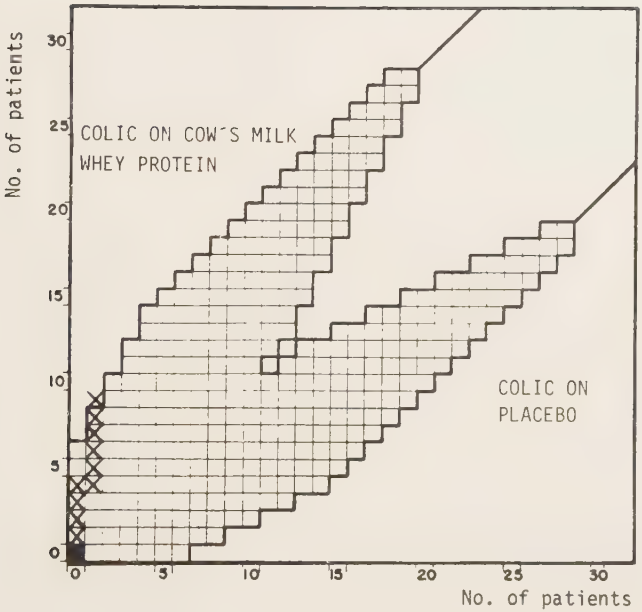

Fig 1. Double-blind cross-over trial of the effect of cow's milk whey protein to 10 mothers, on appearance of colic in their breast-fed infants.

that in one in three of the infants the colic is related to the mother´s cow´s milk consumption. This was further proved by the double-blind cross-over trial.

With the type of statistical method used here, it is of importance that the substances are handled, coded, and the code broken by an independent assessor. The code should not be broken until a certain number of patients, decided at the start of the study, have participated.

According to the sequential plan used here[7], it would mean an important evidence of a connection between whey proteins and infantile colic if 21% of the infants got colic on whey proteins and 10% got colic on placebo. Our corresponding figures were 90% and 10%, respectively.

The double-blind trial showed that capsules containing whey proteins, corresponding to the amount in 80 ml of cow´s milk, caused infantile colic. Whey proteins were chosen because it is well known that the whey proteins, especially β-lactoglobulin, are the most allergenic of the cow´s milk proteins[12, 13].

Our results contradict those of Evans et al.[6] These authors studied 20 breast-fed infants with infantile colic. According to our results the colic could be expected to depend on cow´s milk in only 6 or 7 of these infants. The most important reason that Evans et al.[6] did not find any effect of cow´s milk elimina-

tion may be their choice of soy milk as placebo. A placebo substance should be a substance of no importance as an allergen in infancy. Soy protein is an important allergen in infancy; about one-third of cow´s milk protein intolerant infants are allergic to soy[10, 11] and soy protein has been reported to be as antigenic as cow´s milk proteins[14]. Recently we[15] could demonstrate in a double-blind cross-over study of infantile colic in formula-fed infants that 53% of the infants reacted to soy formula as well as to cow´s milk formula. Only 18% became free of symptoms on soy formula but not on cow´s milk formula. Thus even soy can be an important factor causing infantile colic.

The factor(s) in cow´s milk appearing in human milk and producing colic in the infants has not yet been identified. By double gel diffusion technique and immuno-electrophoresis, precipitates have been formed between fresh human milk and rabbit-antibodies to α-lactalbumin and β-lactoglobulin[16]. These precipitates disappear after freezing the milk. These findings agree with those of Hemmings (personal communication). In the lactating rat dietary proteins as bovine IgG[17] have been demonstrated in the rat milk. With passive cutaneous anaphylaxis, wheat antigen[18] and cow´s milk antigen[19] have been demonstrated in human milk.

In conclusion, infantile colic in breast-fed infants are in 1/3 of the infants related to cow´s milk consumption by the mother. We suggest a diet free of cow´s milk for the mother as a first trial of treatment of infantile colic in breast-fed infants.

ACKNOWLEDGEMENT

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IN VITRO DIGESTION OF COW´S MILK PROTEINS BY DUODENAL JUICE FROM INFANTS WITH VARIOUS GASTROINTESTINAL DISORDERS

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ABSTRACT

The hydrolysis of bovine alpha-lactalbumin, beta-lactoglobulin, and casein by duodenal juice from 20 infants (18 with normal exocrine pancreatic function, 2 with pancreatic insufficiency), aged 3 to 19 months was studied in vitro with the aid of electroimmunoassay and Sodium Dodecyl Sulphate - polyacrylamide gel electrophoresis. The results from the two methods were almost identical. Duodenal juice from infants with cow´s milk protein intolerance (7), celiac disease (5), and unclassified gastrointestinal disorder (6) had the same capacity to hydrolyse the milk proteins. No hydrolysis occurred in the two patients with pancreatic insufficiency. The hydrolysing capacity was not correlated with age in the actual age group. The hydrolysis of the milk proteins occurred at a considerably slower rate when the proteins were crude, as in cow´s milk, adapted, or unadapted formula, than when they were in a purified form. About 1 mg of purified alpha-lactalbumin or beta-lactoglobulin, and about 30 mg of purified casein could be hydrolysed per ml duodenal juice per minute. Corresponding figures for the hydrolysis of the various proteins in cow´s milk were 0.03, 0.12, and 16.1 mg/ml duodenal juice/minute. Preincubation (60 minutes) with gastric aspirate after adjusting pH to 4-5 did not change the results. In conclusion the duodenal juice from infants with normal exocrine pancreatic function has a great

ability to hydrolyse casein. Corresponding hydrolytic capacity for alpha-lactalbumin or beta-lactoglobulin is considerably lower.

Key words: duodenal juice, alpha-lactalbumin, beta-lactoglobulin, casein, cow´s milk protein intolerance

INTRODUCTION

The cow´s milk proteins alpha-lactalbumin, beta-lactoglobulin, and casein are the most commonly used substitutes for human milk proteins. Thus, it is remarkable that there is so little information about the capacity of the infants´ gastrointestinal tract to digest bovine alpha-lactalbumin and beta-lactoglobulin. Sparse information is available in the literature on casein [1-3].

The bovine whey proteins alpha-lactalbumin and beta-lactoglobulin are of high nutritional quality; they are also important allergens in infancy, beta-lactoglobulin is reported to be the most important [4-6].

Immaturity of the gastrointestinal tract, i.e. inadequate proteolytic activity, immature local immunological systems, and increased permeability for macromolecules (antigens) have been proposed as possible causes [7-9] for the relatively large prevalence of cow´s milk protein intolerance in infancy (incidence in Swedish infants = 1.9% [10]).

This investigation studied the capacity of infants´ duodenal juice to hydrolyse bovine alpha-lactalbumin, beta-lactoglobulin, and casein. The duodenal juice was collected from infants with various gastrointestinal disorders, including cow´s milk protein intolerance.

The influence of the gastric juice on the digestability of the three proteins was also studied. The hydrolysis of the three proteins was studied in their purifed form, in cow´s milk, and in adapted and unadapted infant formulas.

PATIENTS

Twenty infants (8 boys, 12 girls), aged 5 days to 19 months were studied (Table I). They were admitted to the hospital because of poor weight gain and stool problems. The examination programme included small intestinal biopsy, duodenal juice aspiration and fecal microscopy [11]. Pancreatic function was assessed by determination of amylase and trypsin activities and agarose gel electrozymogram of duodenal juice [12].

The infants were divided into the following diagnostic groups (Table I):

Cow´s milk protein intolerance: Seven infants with the symptoms disappearing after elimination of cow´s milk from the diet and return of the identical symptoms at two milk challenges. The milk challenges were performed as described previously [10]. Lactose intolerance was excluded.

Celiac disease: Five infants. The diagnosis was based on a flat intestinal mucosa on a gluten containing diet,

TABLE I

CLINICAL MATERIAL

Diagnostic group	*Patient*	*Age* [a] *(months)*	*Trypsin* [b] *(µg/ml)*	*Mucosal histology* [c]
Cow's milk protein intolerance	J.H. ♀	19	940	SL
	H.N. ♂	7	510	N
	M.J. ♀	3	930	S
	S.P. ♀	11	775	N
	A.P. ♂	11	245	N
	K.P. ♀	8	550	SL
	E.C. ♂	6	230	SL
Celiac disease	C.S. ♀	8	592	S
	K.B. ♀	9	675	S
	L.I. ♂	10	574	S
	H.S. ♀	10	495	S
	R.M. ♀	10	400	S
Unclassified gastrointestinal disorder	L.E. ♀	13	380	N
	J.M. ♀	6	545	-
	V.M. ♀	6	350	N
	J.P. ♂	9	365	N
	J.Å. ♂	11	680	N
	P.G. ♂	10	697	-
Pancreatic insufficiency	C.R. ♂	5/30	0	-
	E.M. ♀	17	24	-

a) Age when duodenal juice was collected.

b) Trypsin content > 150 µg/ml is considered as normal [11, 12].

c) N = normal; SL = slight damage; M = moderate damage; S = severe damage.

histological and clinical improvement on a gluten free diet, and histological and/or clinical relapse on a gluten containing diet.

Unclassified gastrointestinal disorders: Six infants with failure to thrive, vomiting, and/or diarrhea for more than 3 weeks when examined. Normal laboratory tests. Symptoms disappeared within a few months.

Pancreatic insufficiency: Two infants with cystic fibrosis - the diagnosis based upon abnormal sweat tests (sodium values 87 and 128 mmol/l) and typical clinical course.

The study was approved by the Ethical Committee of the University of Lund.

MATERIAL

Purified preparations of alpha-lactalbumin and beta-lactoglobulin (generous gifts from Nestlé laboratory, Switzerland); alpha-casein (C 7891), Sigma Chemicals, St Louis, USA); pasteurised standardized cow´s milk; two adapted formulas (casein/whey ratio = 40/60), Milkotal® (Findus AB, Sweden) and Baby Semp I® (Semper AB, Sweden) and one unadapted infant formula (casein/whey ratio = 80/20), Enfamil® (Mead Johnson, USA); Diisopropyl-fluorophosphate (DFP) (Merck, Darmstadt, West-

Germany). Rabbit antibodies against alpha-lactalbumin, beta-lactoglobulin, and casein were raised in our laboratory. Acrylamide, NN'-methylene-bisacrylamide (BDH Chemicals Ltd, Poole, England), N, N, N', N'-tetramethylethylendiamine (E. Merck, Darmstadt), sodiumdodecylsulphate (SDS), low molecular weight standards for polyacrylamide gel electrophoresis, and electrophoresis equipment model 220 vertical slab electrophoresis cell (Bio Rad Lab, California, USA). All other chemicals were of analytical grade.

METHODS

Immunization procedure

100 µg (in 0.5 ml 0.9% saline) of alpha-lactalbumin, beta-lactoglobulin, and casein respectively, were mixed with an equal amount of Freund´s complete adjuvant (totally 1 ml) and injected intracutaneously, subscapularly into adult rabbits. Booster shots were given after 4 and 8 weeks, thereafter the serum was collected and IgG fractions isolated [13].

Collection of duodenal juice

The infants were fasted for 4 hours. A double-lumen tube (Salem sump tube - Sheridan) was placed with the tip in the ascending part of the duodenum (fluoroscopic

control). The juice was collected on crushed ice and was analysed either directly or after storage at -20°C.

Gastric aspirates

Aspirates were drawn from infants fed nasogastrically because of cerebral damage. The aspirates were tested for pH and for proteolytic activity with a single radial digestion technique: 0.1% casein in 2.25% agarose and 0.2 N Na-acetate-HCl, pH 2.0. After incubation (37°C, 3 h), the slide was immersed in 2% acetic acid (1 h), dried, and stained with Coomassie Brilliant Blue R 250.

Substrate solutions

Hydrolysis of alpha-lactalbumin and beta-lactoglobulin: 1) 0.9 mg of alpha-lactalbumin or beta-lactoglobulin dissolved in 450 µl phosphate buffer pH 7.35; 2) alpha-lactalbumin, beta-lactoglobulin, and casein mixed in phosphate buffer, pH 7.35, in the same proportions as in cow´s milk, with a total protein content of 0.9 mg/450 µl; 3) standardized cow´s milk or infant formula diluted with phosphate buffer, pH 7.35, to give a total protein content of 0.9 mg/450 µl; 4) bovine serum (diluted 1:4 with 0.9% NaCl and preheated 56°C for 30 min) containing alpha-lactalbumin, beta-lactoglobulin, and casein in the same proportions as in cow´s milk (total protein content ~1.5 mg/450 µl).

Hydrolysis of casein: 1) 11.3 mg of casein dissolved in 450 µl phosphate buffer pH 7.35; 2) 450 µl undiluted cow´s milk or infant formula.

Procedure of hydrolysis

The substrate (450 µl) was incubated with 50 µl duodenal juice at 37°C with continuous stirring. Aliquots (10-50 µl) were collected at different times and were mixed with an appropriate volume of ice cooled distilled water for electroimmunoassay or with an equal volume of the anionic detergent sodium dodecyl sulphate (SDS 2%) for SDS-polyacrylamide gel electrophoresis. To obtain a background value 90 µl of substrate was mixed with 10 µl boiled (5 min) duodenal juice.

Preincubation with gastric juice

The pH of the gastric aspirates varied from 1.5 to 2.0. Two mg of alpha-lactalbumin or beta-lactoglobulin, 20 mg of casein (dissolved in phosphate buffer pH 7.35) or 450 µl of cow´s milk was incubated with 180-200 µl gastric aspirate at 37°C during 30-60 minutes at pH 1.5-2.0 or at pH 4.5-5.0, which was attained by adding 0.4 M NaOH. Incubation was stopped by adding 0.4 M NaOH, raising pH to 7.5-8.0. The duodenal juice was added to the digestion mixture continuing the hydrolysis as described above.

Determination of the hydrolysis

Electroimmunoassay was done according to Laurell [14] in 0.8% agarose with 3% polyethylene glycol. A barbital-sodium-barbital buffer pH 8.6 containing 0.002 M calcium lactate was used. The antibody concentration in the gel was 1.5-5%.

SDS-polyacrylamide gel electrophoresis. Reagents and gels were used according to the Laemmli system [15]. Gels containing 3% (stacking gel) and 13.5% (separating gel) acrylamide were prepared. Electrophoresis was carried out starting with 10 mA per gel. When the dye front entered the separating gel the current was raised to 100 mA per gel. Total running time 4-5 h; staining with Coomassie Brilliant Blue R 250; amount of protein measured by soft laser densitometer.

Statistical evaluation

For comparisons of groups, Student´s t-test and the non-parametric Mann Whitney´s test were used.

RESULTS

Method

Figs 1 and 2 illustrate the hydrolysis of the various proteins visualized by electroimmunoassay and by SDS-

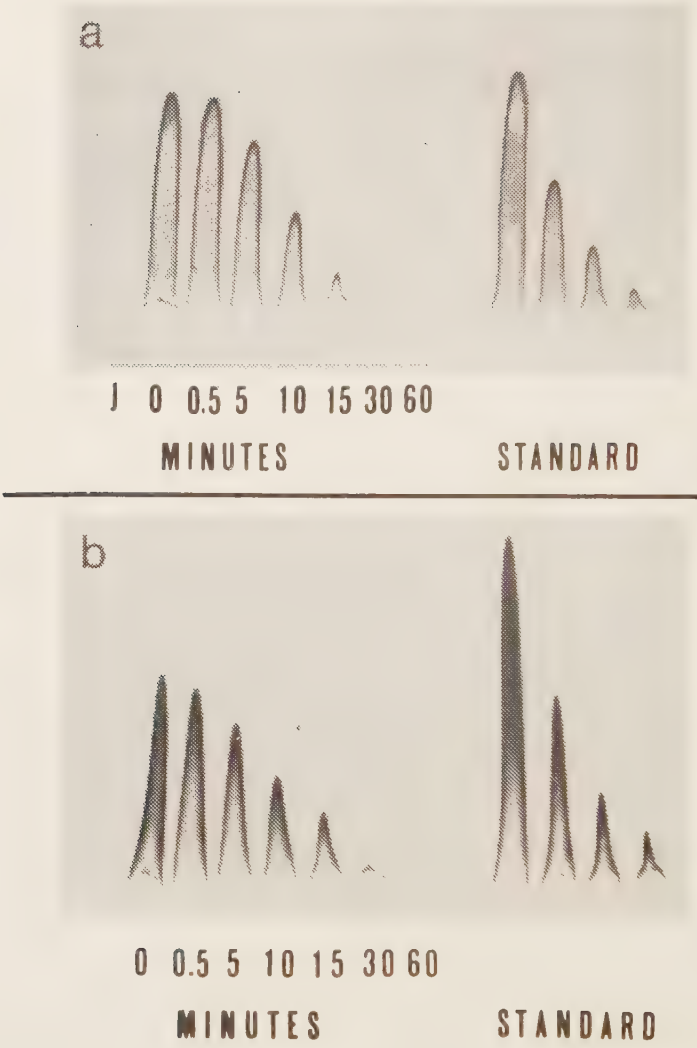

Fig 1. Electroimmunoassay of α-lactalbumin and β-lactoglobulin hydrolysed by duodenal juice from a 3 months infant, with cow´s milk protein intolerance. Running time 4 h, 20 v/cm. Stained with Coommassie Brilliant Blue R 250. Minutes = time of incubation with duodenal juice.

a) α-lactalbumin. Dilution of standard 1/1 (=0.0025%), 1/2, 1/4, 1/8. J = duodenal juice.

b) β-lactoglobulin. Dilution of standard 1/1 (=0.01%), 1/2, 1/4, 1/8.

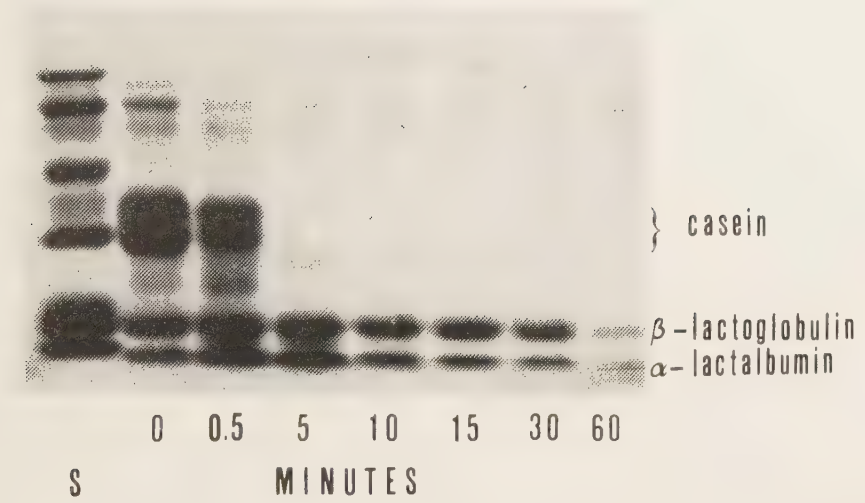

Fig 2. SDS-polyacrylamide gel electrophoresis of standardized cow´s milk hydrolysed by duodenal juice from a 3 months infant, with cow´s milk protein intolerance. S = low molecular weight standard for polyacrylamide gel electrophoresis with an operating range of 15,000 - 100,000 Daltons. Minutes = time of incubation with duodenal juice. Anode: bottom.

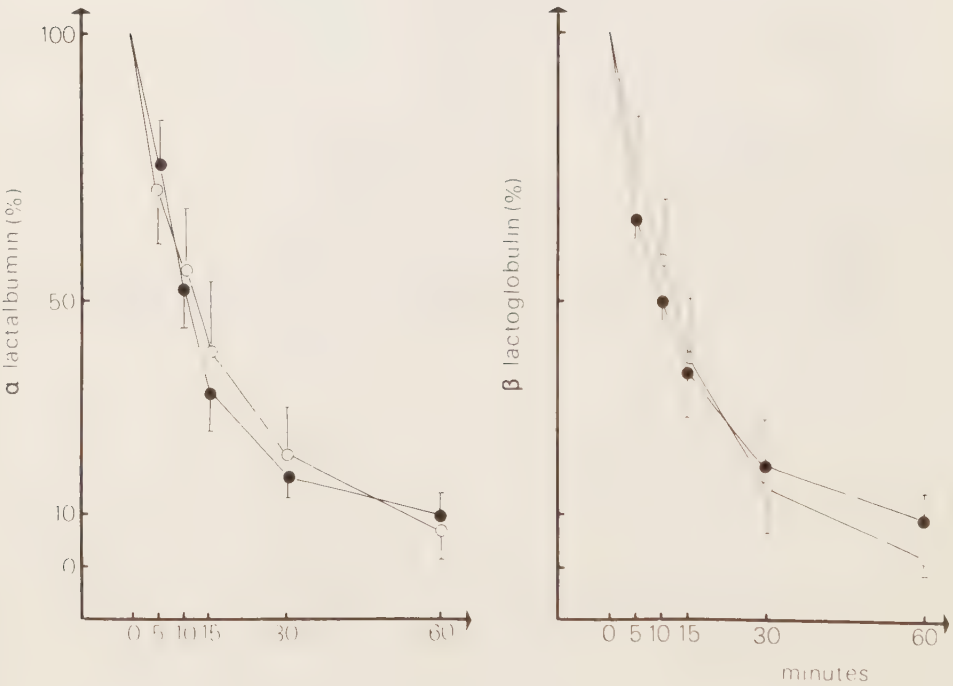

Fig 3. Hydrolysis of purified α-lactalbumin (n = 8) and β-lactoglobulin (n = 10) by duodenal juice (50 µl, see Methods), followed by electroimmunoassay (○-○) and SDS-polyacrylamide gel electrophoresis (●-●). 100% corresponds to 0.8 - 1.0 mg of protein. Mean values ± SEM.

polyacrylamide gel electrophoresis, respectively. Fig 3 compares the two methods. Identical results were obtained for alpha-lactalbumin or beta-lactoglobulin. Casein gave several precipitates on electroimmunoassay (2 to 3 anodal peaks), making it impossible to determine the capacity of the duodenal juice to hydrolyse casein with this method. For this purpose SDS-polyacrylamide gel electrophoresis was used.

The efficiency of ice-cooled water to stop the hydrolysis was controlled by using 0.01 mol/1 DFP. Hydrolysis continued about 10% further when ice-cooled water was used.

Freezing (-20^{o}C) of the duodenal juice did not affect its hydrolysing capacity; juice from one infant was examined directly after collection, after 3 months and after 6 months of freezing. In agreement with our previous experience [16] the trypsin activity was the same at the three occasions, as was the capacity to hydrolyse the cow's milk proteins.

Clinical material

Fig 4 shows the hydrolysis, measured by electroimmunoassay, of purified alpha-lactalbumin by duodenal juice from the infants in the various diagnostic groups. No hydrolysis occurred in the two patients with pancreatic

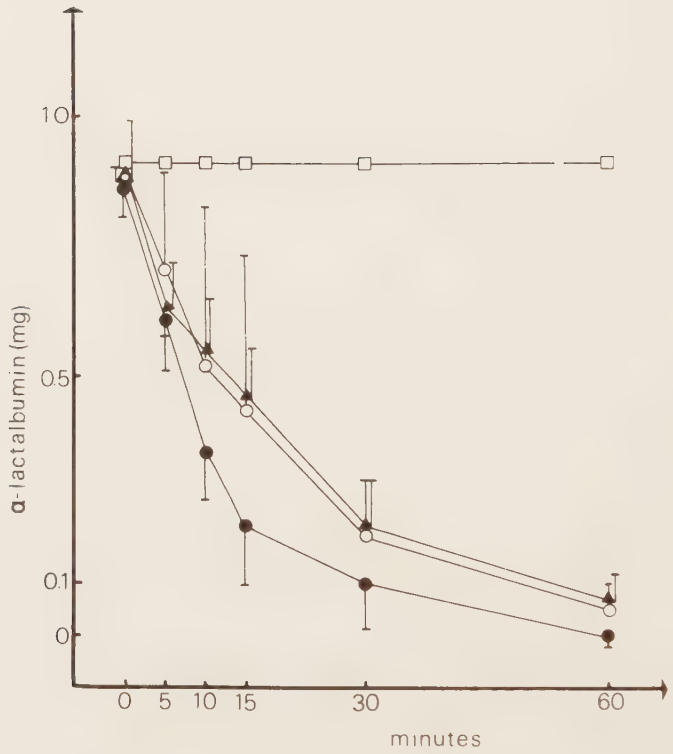

Fig 4. Hydrolysis of purified α-lactalbumin by duodenal juice (50 µl, see Methods) from infants with cow's milk protein intolerance (▲-▲) (n = 7); celiac diasese (○-○) (n = 3); unclassified gastro-intestinal disorder (●-●) (n = 4); cystic fibrosis (□-□) (n = 2). Mean values ± SEM.

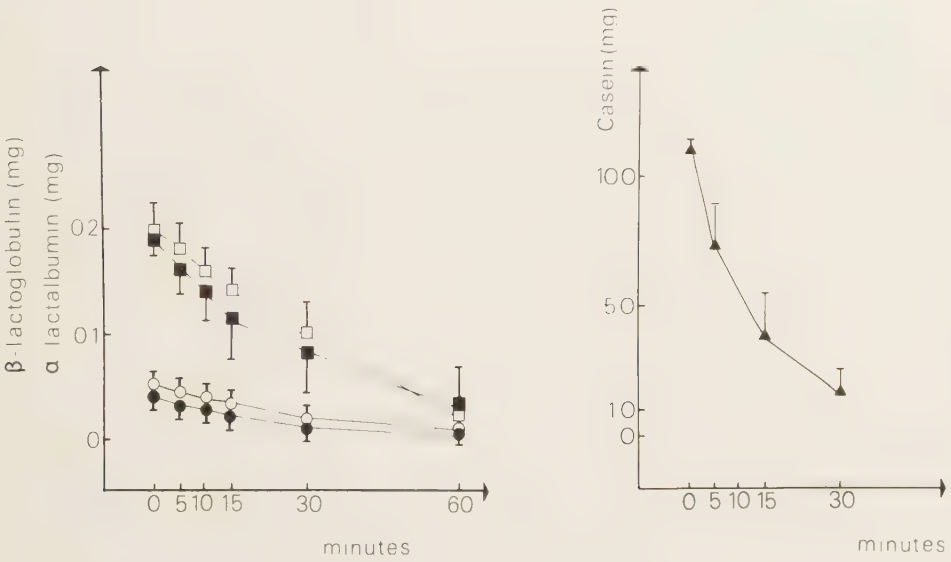

Fig 5. Hydrolysis of α-lactalbumin (○-○, ●-●), β-lactoglobulin (□-□, ■-■) and casein (▲-▲) in cow's milk by duodenal juice (50 µl, see Methods) from infants with cow's milk protein intolerance (□-□, ○-○) (n = 5); other gastro-intestinal disorders (■-■, ●-●) (n = 7). For casein these two diagnostic groups have been combined (n = 6). Hydrolysis of α-lactalbumin and β-lactoglobulin measured by electroimmunoassay, hydrolysis of casein measured by SDS-polyacrylamide gel electrophoresis. Mean values ± SEM.

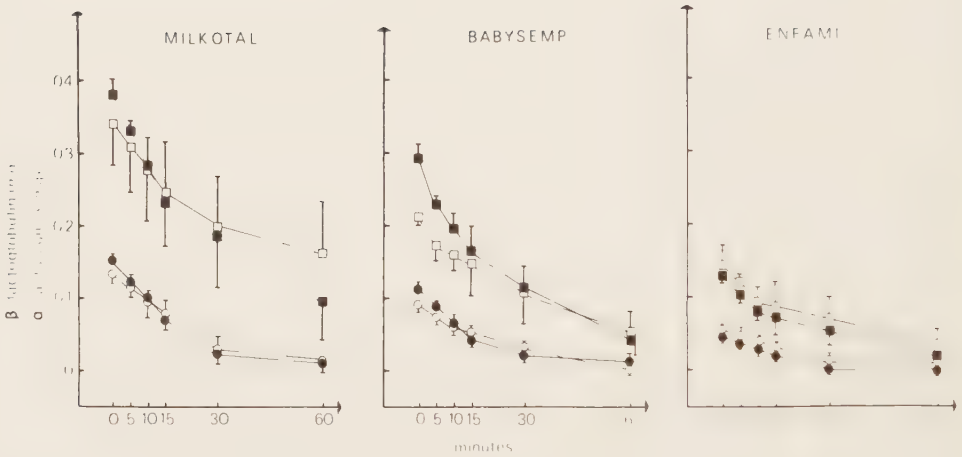

Fig 6. Hydrolysis of α-lactalbumin (○-○, ●-●) and β-lactoglobulin (□-□, ■-■) in 3 infant formulas, by duodenal juice (50 µl, see Methods) from infants with cow's milk protein intolerance (□-□, ○-○) (n = 4) or other gastrointestinal disorders (■-■, ●-●) (n = 5). Mean values ± SEM.

insufficiency. Between the other three groups there were no significant differences ($p>0.05$). Corresponding results were found with purified beta-lactoglobulin and with casein. The capacity to hydrolyse alpha-lactalbumin and beta-lactoglobulin was not correlated with age.

The hydrolysis of the proteins in standardized cow´s milk is demonstrated in Fig 5 and in the three infant formulas in Fig 6. There were no significant differences between the diagnostic groups ($p>0.05$).

From the Figs 4, 5, and 6, it is evident that the rate of hydrolysis of the various proteins is slower when the substrate solution is crude as in cow´s milk or infant formulas. To study the influence of the composition of the substrate solution on the rate of hydrolysis, alpha-lactalbumin, beta-lactoglobulin, and casein were mixed in phosphate buffer or in bovine serum. The hydrolysis of alpha-lactalbumin and beta-lactoglobulin in these mixtures was determined. Fig 7 compares the hydrolysis of alpha-lactalbumin in the various substrate solutions. $T\frac{1}{2}$ (= time required to hydrolyse 50% of the protein) for alpha-lactalbumin was 5 minutes when present as a single protein or together with beta-lactoglobulin and casein in a phosphate buffer solution. $T\frac{1}{2}$ for alpha-lactalbumin in bovine serum was 12 minutes and in cow´s milk 37 minutes. Corresponding results for beta-lactoglobulin were 8, 10, 19 and 25 minutes respectively.

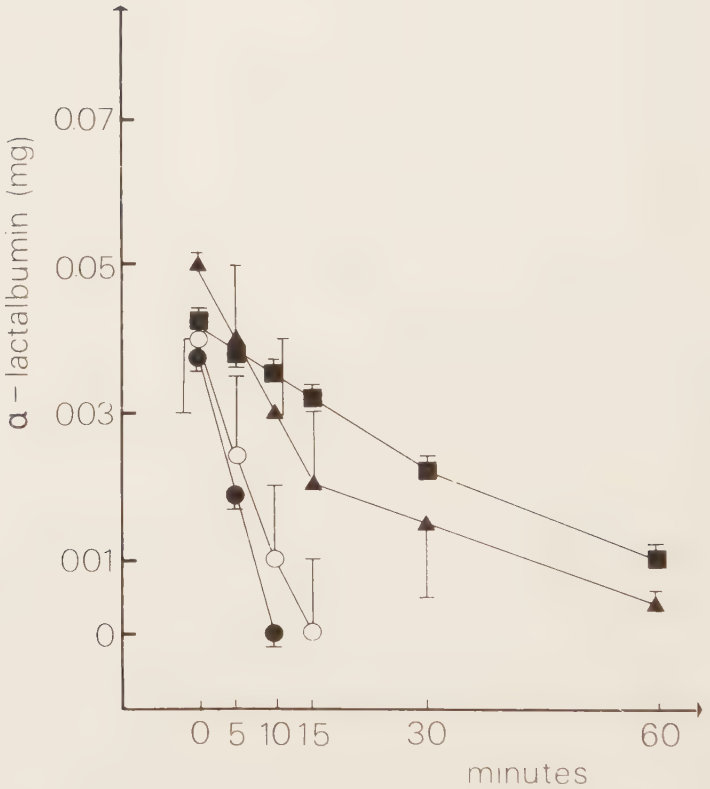

Fig 7. Hydrolysis of α-lactalbumin in various protein solutions, by duodenal juice (50 µl, see Methods) from infants. Phosphate buffer solution (pH 7.35) containing α-lactalbumin (1.5 mg/ml) (○-○) (n = 2); phosphate buffer solution (pH 7.35) containing α-lactalbumin (1.5 mg/ml), β-lactoglobulin (3.5 mg/ml), casein (25 mg/ml) (●-●) (n = 5); bovine serum, to which were added α-lactalbumin (1.5 mg/ml), β-lactoglobulin (3.5 mg/ml), casein (25 mg/ml) (▲-▲) (n = 3); cow´s milk (■-■) (n = 12). Mean values ± SEM.

On average one ml of duodenal juice hydrolysed 0.84 mg of purified alpha-lactalbumin, 0.94 mg of purified beta-lactoglobulin and 29.3 mg of purified casein per minute (Table II). Table II also demonstrates that the capacity of duodenal juice to hydrolyse the proteins in purified form exceeded the capacity to hydrolyse them in cow´s milk by about 30 times for alpha-lactalbumin, by 10 times for beta-lactoglobulin and twice for casein.

Preincubation with gastric aspirate at pH 1.5 showed a considerable hydrolysis of the various proteins. With 180 µl of gastric aspirate 2 mg of protein was completely hydrolysed in 30 minutes. At pH 4.5 there was no detectable hydrolysis of the various proteins in purified form or in cow´s milk after incubation during 60 minutes. The subsequent incubation with duodenal juice gave the same hydrolytic rates as without gastric preincubation (n = 4).

DISCUSSION

To the best of our knowledge this is the first study investigating the luminal phase of the breakdown of the cow´s milk proteins alpha-lactalbumin and beta-lactoglobulin, in the upper part of the gastrointestinal tract, during infancy. It has been shown in the classic

TABLE II

CAPACITY OF INFANTS' DUODENAL JUICE TO HYDROLYSE α-LACTALBUMIN, β-LACTOGLOBULIN AND CASEIN (MG PROTEIN/ML DUODENAL JUICE/MINUTE). MEAN VALUES ± SEM (N).

Substrate / Diagnosis	Cow's milk protein			Human milk		
	α-lactalbumin [a]	β-lactoglobulin [a]	casein [b]	α-lactalbumin [a]	β-lactoglobulin [a]	casein [b]
Cow's milk protein intolerance	0.76 ± 0.18 (7)	1.16 ± 0.20 (6)	14.0, 33.6	0.03 ± 0.01 (5)	0.15 ± 0.05 (5)	13.6, 27.2
Celiac disease	0.78 ± 0.41 (3)	0.82 ± 0.20 (3)	35.6, 26.4	0.02 ± 0.01 (3)	0.11 ± 0.03 (3)	6.0, 15.0
Unclassified gastrointestinal disorder	1.03 ± 0.28 (4)	0.70 ± 0.16 (4)	34.4, 32.0	0.03 ± 0.01 (4)	0.09 ± 0.04 (4)	16.8, 18.2
Totally	0.84 ± 0.14 (14)	0.94 ± 0.12 (13)	29.3 ± 3.3 (6)	0.03 ± 0.01 (12)	0.12 ± 0.03 (12)	16.1 ± 4.2 (6)

a) Measured by electroimmunoassay and SDS-polyacrylamide gel electrophoresis.

b) Measured by SDS-polyacrylamide gel electrophoresis. Individual observations are given.

studies by Borgström et al. [17] that 30-40% of the protein is digested and absorbed in the upper part of jejunum in infants.

With the two methods used in the present study, we have followed the initial steps of the digestion catalyzed by the endopeptidases pepsin, trypsin, chymotrypsin and elastase.

In electroimmunoassay the basic principle of quantitative estimation of proteins by precipitation with the corresponding antibodies is used. Theoretically, parts of a molecule can be split off without interfering with its antigenic sites. The other method, SDS-polyacrylamide gel electrophoresis, separates the proteins according to the molecular mass of their polypeptide chains [18, 19]. The staining intensity of the bands corresponds to the concentration of the peptide chains.

A comparison between the two methods, when hydrolysing alpha-lactalbumin or beta-lactoglobulin with duodenal juice, showed almost identical results. From this we may conclude that during the first phase of the hydrolysis these two proteins also lose their ability to form precipitates with their corresponding antibodies.

Electroimmunoassay of casein did not allow quantita-

tion of the hydrolysis of this protein, but it was evident that the antigenicity of casein to its corresponding antibody disappeared even faster than that of alpha-lactalbumin and beta-lactoglobulin.

Medical ethics precludes the study of normal infants. All the infants in this study had some gastrointestinal complaints. Their exocrine pancreatic function was normal in 18/20 as judged by the pancreatic enzyme content of the duodenal juice [12].

These infants´ age ranged from 3 to 19 months with a mean age of 9 months (Table I). However, the hydrolysing capacity was not found to be age related in the actual age group. This is in agreement with the fact that proteolytic enzymes are well developed already at 1 month of age [20].

No differences were found between the 3 diagnostic groups; cow´s milk protein intolerance, celiac disease or unclassified gastrointestinal disorder, in their capacity to hydrolyse the three proteins. Thus the infants with cow´s milk protein intolerance were able to hydrolyse cow´s milk proteins as efficiently as the other infants. This does not support the theory that a defect in proteolytic activity of the duodenal juice contributes to the pathogenesis of cow´s milk protein intolerance.

The possibility still exists that later steps of the hydrolysis of these proteins are defective. The final step, involving intestinal dipeptidases, is not primarily affected in infants with cow´s milk protein intolerance [21].

Digestive products of bovine milk proteins have been shown to have an antigenic specificity which differs from the parent proteins, as judged by sensitization of guinea pig uterus and production of precipitins [22]. In 1942 Cooke [23] reported that sensitization to digestive derivatives of cow´s milk proteins existed and that in some cases delayed clinical reactions could be explained on this basis. In 1953 [24] this hypothesis was tested but could not be substantiated. Haddad et al. [25] demonstrated IgE antibodies in humans to enzymatic digests of βLG and suggested that the reactivity to these antigens might be greater than to the native protein in certain individuals. Recently Schwartz et al. [26] investigated the occurrence of specific IgE antibodies to milk proteins and new antigens generated by in vitro pepsin hydrolysis of βLG in patients with suspected hypersensitivity to cow´s milk. The findings suggested that undigested milk proteins were more reactive than digested proteins.

Only few studies have attempted to quantitate the capacity of intestinal juice to digest food proteins. Coldberg et al. [27] reported that several kg of casein could be digested daily by ileal drainage fluid from adults. Hirata et al [2] studied infants´ capacity to digest casein *in vivo* by intubation of the ileum. They reported that, e.g., a 5 month old infant could utilize about 4 g casein in a meal. Using the similar *in vitro* model as in the present study, Lindberg [3] found that infants´ duodenal juice hydrolysed on average 10 mg of casein into trichloracetic acid soluble peptides per ml duodenal juice per minute, a lower figure than we found in this study, but differences in methods of following the hydrolysis and also type of casein may explain this discrepancy. Alpha-casein, used in this study, is said to be more easily digested than total casein used in Lindberg´s previous study. Both studies show that the infants´ duodenal juice has a very large ability to hydrolyse casein into smaller paptides. The corresponding hydrolytic capacity for alpha-lactalbumin and beta-lactoglobulin is considerably lower. Preincubation with gastric juice at pH 4.5, the pH in the stomach during more than 60 minutes after a milk meal [28], did not affect the hydrolysis by duodenal juice in this in vitro model.

The present investigation, performed in vitro without quantitative collection of duodenal juice, does not

allow calculation of the absolute amount of alpha-lactalbumin, beta-lactoglobulin, or casein that can be hydrolysed by the duodenal juice per hour. However, if the figures for volume of duodenal juice reported by Hadorn et al. [29] for children (about 4 ml/kg body weight/hour) are used, the following values are obtained (Table II; capacity to hydrolyse the various proteins in cow´s milk): 7.2 mg of alpha-lactalbumin, 28.8 mg of beta-lactoglobulin, and 3.8 g of casein could be hydrolysed per kg body weight and hour. This means that the duodenal juice from an infant weighing 7 kg is able to hydrolyse about 50 mg alpha-lactalbumin, 200 mg beta-lactoglobulin, and 25 g casein per hour, values equal to about 35 ml, 60 ml, and 1000 ml of cow´s milk, respectively.

The hydrolysis of alpha-lactalbumin or beta-lactoglobulin occurred at a considerably slower rate when present in crude form, as in cow´s milk and in adapted or unadapted formula, than in purified form. The reason for this discrepancy is obscure. A mixture of purified alpha-lactalbumin, beta-lactoglobulin, and casein in phosphate buffer did not affect the rate of hydrolysis of the individual proteins. But when the three proteins were mixed in bovine serum, the hydrolysis occurred at a slower rate but not as slow as in cow´s milk. Thus it seems that some protease inhibiting factor(s) are present in cow´s milk, in formulas and probably in bovine serum.

Possibly the relatively slow hydrolysis of alpha-lactalbumin and beta-lactoglobulin can contribute to their being more allergenic than the more easily digestible casein [4-6]. From this point of view, the suitability to prepare adapted formulas with a casein/whey ratio of 40/60 and even of 30/70 for infants could be questioned.

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VI

PARTIAL HYDROLYSIS OF COW'S MILK PROTEINS BY HUMAN TRYPSINS AND ELASTASE

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Summary

The hydrolysis of bovine α-lactalbumin, β-lactoglobulin, and casein by human anodal and cathodal trypsins and elastase was studied with the aid of electroimmunoassay and sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The rate of hydrolysis of the various proteins by cathodal elastase exceeded that by anodal or cathodal trypsin. Casein was hydrolysed more efficiently than α-lactalbumin or β-lactoglobulin. The hydrolysis of the three proteins occurred at a considerably slower rate when present in crude form, as in cow´s milk, than in purified form.

Introduction

The bovine whey proteins α-lactalbumin and β-lactoglobulin are of high nutritional quality. Together with bovine casein they are the most commonly used substitutes for human milk protein. The bovine whey proteins are also considered important allergens in infancy, β-lactoglobulin is reported to be the most important (1, 2, 3).

Recently we reported (4) that in vitro purified casein was hydrolysed to a greater extent than purified α-lactalbumin and β-lactoglobulin by duodenal juice from infants with various gastrointestinal disorders.

The main endopeptidases of the duodenal juice are trypsin, elastase, and chymotrypsin. The literature has only sparse information on hydrolysis of the bovine proteins α-lactalbumin, β-lacto-

globulin, and casein by trypsin or elastase (5, 6, 7). No information is available concerning hydrolysis by human trypsins or elastases.

This study reports the ability of human anodal and cathodal trypsins and cathodal elastase to hydrolyse bovine α-lactalbumin, β-lactoglobulin, and casein.

Materials and methods

Purified human anodal and cathodal trypsins (8) and cathodal elastase (9) were available in our laboratory. The enzyme stock solutions used contained; cathodal trypsin 1.05-1.54 g/l, anodal trypsin 0.68-0.74 g/l, cathodal elastase 0.16 g/l. Trypsin activity was measured with n-benzoyl-DL-arginine-p-nitroanilide (BAPNA) as substrate (10). Elastase activity was measured with succinyl-trialanine-p-nitroanilide (Suc $(Ala)_3$NA) as substrate (11).

Purified preparations of α-lactalbumin and β-lactoglobulin (generous gifts from Nestlé laboratory, Switzerland); α-casein (C7891, Sigma Chemicals, St Louis, USA); and pasteurised standardised cow's milk were used as substrates. Rabbit antibodies against α-lactalbumin, β-lactoglobulin, and casein were raised in our laboratory (4). 0.8% agarose (Miles Seravac Maidenhead England) in 0.075 mol/l barbital-sodium barbital, pH 8.6, containing 2 mmol/l calcium lactate was used in electroimmunoassay. Acrylamide, NN'-methylene-bisacrylamide (BDH Chemicals Ltd, Poole, England) were used in polyacrylamide gel electrophoresis. All other chemicals were of analytical grade.

Substrate solutions. When hydrolysing with trypsins we used a 0.05 M TRIS 0.02 M $CaCl_2$ buffer, pH 8.2 and with elastase a 0.1 M TRIS buffer, pH 8.0. 0.9 mg of purified α-lactalbumin or β-lactoglobulin, or 11.3 mg of purified casein was dissolved in 450 µl buffer solution. To hydrolyse α-lactalbumin or β-lactoglobulin in standardized cow´s milk, we diluted the milk with buffer solution to give a total protein content of 0.9 mg/450 ul. Hydrolysis of casein in standardised cow´s milk was done with 450 ul undiluted cow´s milk.

The enzyme concentrations of the digestion mixtures were: cathodal trypsin 0.105-0.154 g/l, anodal trypsin 0.068-0.074 g/l, cathodal elastase 0.016 g/l.

The substrate solution (450 ul) was incubated with 50 ul enzyme solution at 37°C during 1/2, 5, 10, 15, 30, 60 minutes (previously described in detail) (4). The rate of the hydrolysis was measured by electroimmunoassay (10) and by SDS (sodium-dodecyl-sulphate)-polyacrylamide gel electrophoresis (13).

Results

Table I reports the results. The rate of hydrolysis of the various proteins by cathodal elastase exceeded that by anodal or cathodal trypsin. Casein was hydrolysed more efficiently than α-lactalbumin or β-lactoglobulin. Casein hydrolysis could not be determined by electroimmunoassay as several precipitates appeared (4).

Hydrolysis of standardised cow´s milk by cathodal trypsin and cathodal elastase showed that the ability to hydrolyse the proteins in

TABLE I

PARTIAL HYDROLYSIS OF PURIFIED α-LACTALBUMIN, β-LACTOGLOBULIN, AND CASEIN BY HUMAN TRYPSINS AND ELASTASE (MG PROTEIN/MG ENZYME/MINUTE)

	α-lactalbumin		β-lactoglobulin		Casein
	Method A *	Method B **	Method A *	Method B **	Method B **
Anodal trypsin (2 experiments)	n.d. ***	0.14	n.d.	0.49	-
	n.d.	0.25	n.d.	0.60	3.67
Cathodal trypsin (3 experiments)	n.d.	0.15	0.15	0.59	11.2
	0.19	0.08	0.18	0.18	-
	0.10	0.12	0.13	0.77	11.8
Cathodal elastase (3 experiments)	0.40	2.25	1.33	3.0	47.0
	0.60	1.80	1.83	3.8	21.7
	0.60	2.70	1.17	4.3	36.0

* Hydrolysis measured by electroimmunoassay

** Hydrolysis measured by SDS-polyacrylamide gel electrophoresis

*** n.d. = no detectable hydrolysis

**** Hydrolysis of casein was measured by SDS-polyacrylamide gel electrophoresis, only

purified form exceeded the ability to hydrolyse them in cow´s milk by about 10-15 times for β-lactoglobulin and by about 3 times for casein. There was no detectable hydrolysis of α-lactalbumin in cow´s milk.

Discussion

The study shows - as could be anticipated because of the different substrate specificities of the enzymes - that the two trypsins hydrolysed α-lactalbumin, β-lactoglobulin, and casein to a minor degree than cathodal elastase. Jost and Monti (7) found that about 8 per cent of all peptide bonds in native or heat-denaturated whey proteins were hydrolysed by porcine trypsin. According to the lysine and arginine content in the whey proteins, 9.3 per cent of the peptide bonds could theoretically be split by trypsin (7).

Discrepancies of the results were obtained with the two methods used to measure the initial hydrolysis of α-lactalbumin and β-lactoglobulin, i.e. they slowly lost the antigenicity to their corresponding antibodies. This contrasts to the situation when a mixture of enzymes is used, e.g. when using duodenal juice, where the two methods gave identical results (4).

In agreement with the results from our previous study with duodenal juice as enzyme-solution (4), the various enzymes showed considerable ability to hydrolyse casein. The corresponding hydrolytic capacity for α-lactalbumin and β-lactoglobulin was considerably lower. As in the previous study (4) the hydrolysis of α-lactalbumin and β-lactoglobulin occurred at a considerably slower rate when

present in crude form, as in cow´s milk, than in purified form. This might be because, e.g., of the presence of protease inhibitors in cow´s milk or substrate competition. This needs further investigation.

Inadequate proteolytic activity in the gut has been proposed as one of several causes of cow´s milk protein intolerance in infancy (14). The two trypsins are well developed in early infancy (15). As stated above, the tryptic hydrolysis of the bovine whey proteins is limited. Small amounts of cathodal elastase in the duodenal juice was found in early infancy and a continuing development was demonstrated during the first two years of life (16). Borulf and Lindberg (16) found no correlation between low cathodal elastase content in duodenal juice and the diagnosis cow´s milk protein intolerance. However, the infants were studied when they already had a manifest cow´s milk protein intolerance. We have no information on content of cathodal elastase in their duodenal juice at the time (i.e. the first months of life) when they were sensitized to the cow´s milk proteins.

In duodenal juice from infants below 7 months of age, the elastase esterolytic activity (substrate: Suc $(Ala)_3NA$) was mainly derived from an enzyme not reacting with antibodies to cathodal elastase (16), probably anodal elastase. Its contribution to the proteolytic capacity of duodenal juice in early infancy is still to be established. However, it seems from preliminary studies in our laboratory that purified human anodal elastase does not hydrolyse the bovine whey proteins as efficiently as cathodal elastase.

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